

Morphogen-Induced Nerve Regeneration and Repair

5

CROSS REFERENCE TO RELATED APPLICATIONS

See 51
This application is a continuation-in-part of USSN 752,764 and copending USSN 753,059, both filed August 30, 1991 and both continuations-in-part of USSN 667,274, filed March 11, 1991.

BACKGROUND OF THE INVENTION

15 The present invention relates to methods for enhancing the survival of neuronal cells in vivo and to methods, compositions and devices for maintaining neural pathways in vivo. More particularly, the invention provides methods for enhancing survival of neuronal cells at risk of dying, including methods for redifferentiating transformed cells of neural origin and methods for maintaining phenotypic expression of differentiated neuronal cells. The invention also provides means for repairing damaged neural pathways, including methods for stimulating axonal growth over extended distances, and methods for alleviating immunologically-related nerve tissue damage. In a particular embodiment of the invention, this invention provides a method for stimulating cell adhesion molecule expression in cells, and particularly nerve cell adhesion molecule expression in neurons. Finally, the invention provides means for evaluating nerve tissue stasis and identifying neural dysfunction in a mammal.

35

08937755-092597

25 During development, differentiating neurons from
the central and peripheral nervous systems send out
axons that must grow and make contact with specific
target cells. In some cases, growing axons must cover
enormous distances; some grow into the periphery,
30 whereas others stay confined within the central nervous
system. In mammals, this stage of neurogenesis is
complete during the embryonic phase of life and
neuronal cells do not multiply once they have fully
differentiated.

Accordingly, the neural pathways of a mammal are particularly at risk if neurons are subjected to mechanical or chemical trauma or to neuropathic degeneration sufficient to put the neurons that define the pathway at risk of dying. A host of neuropathies, some of which affect only a subpopulation or a system of neurons in the peripheral or central nervous systems have been identified to date. The neuropathies, which may affect the neurons themselves or the associated glial cells, may result from cellular metabolic dysfunction, infection, exposure to toxic agents, autoimmunity dysfunction, malnutrition or ischemia. In some cases the cellular dysfunction is thought to induce cell death directly. In other cases, the neuropathy may induce sufficient tissue necrosis to stimulate the body's immune/inflammatory system and the mechanisms of the body's immune response to the initial neural injury then destroys the neurons and the pathway defined by these neurons.

Currently no satisfactory method exists to repair the damage caused by these neuropathies, which include multiple sclerosis, amyotrophic lateral sclerosis (ALS), Huntington's chorea, Alzheimer's disease, Parkinson's disease (parkinsonism), and metabolically derived disorders, such as hepatic encephalopathy. Current attempts to counteract the effects of severe traumatic or neural degenerative lesions of the brain and/or spinal cord have to date primarily involved implantation of embryonic neurons in an effort to replace functionally, or otherwise compensate for, lost or deficient neurons. Currently, however, human fetal cell transplantation research is severely restricted. Administration of neurotrophic factors such as nerve growth factor and insulin-like growth factor also have

20250957680

Administration of neurotrophic factors to the CNS

Where the damaged neural pathway results from CNS axonal damage, autologous peripheral nerve grafts have been used to bridge lesions in the central nervous system and to allow axons to make it back to their normal target area. In contrast to CNS neurons, neurons of the peripheral nervous system can extend new peripheral processes in response to axonal damage. This regenerative property of peripheral nervous system axons is thought to be sufficient to allow grafting of these segments to CNS axons. Successful grafting appears to be limited, however, by a number of factors, including the length of the CNS axonal lesion to be bypassed, and the distance of the graft sites from the CNS neuronal cell bodies, with successful grafts occurring near the cell body.

Within the peripheral nervous system, this cellular regenerative property of neurons has limited ability to repair function to a damaged neural pathway.

Specifically, the new axons extend randomly, and are
35 often misdirected, making contact with inappropriate

targets that can cause abnormal function. For example, if a motor nerve is damaged, regrowing axons may contact the wrong muscles, resulting in paralysis. In addition, where severed nerve processes result in a gap of longer than a few millimeters, e.g., greater than 10 millimeters (mm), appropriate nerve regeneration does not occur, either because the processes fail to grow the necessary distance, or because of misdirected axonal growth. Efforts to repair peripheral nerve damage by surgical means has met with mixed results, particularly where damage extends over a significant distance. In some cases, the suturing steps used to obtain proper alignment of severed nerve ends stimulates the formulation of scar tissue which is thought to inhibit axon regeneration. Even where scar tissue formation has been reduced, as with the use of nerve guidance channels or other tubular prostheses, successful regeneration generally still is limited to nerve damage of less than 10 millimeters in distance. In addition, the reparative ability of peripheral neurons is significantly inhibited where an injury or neuropathy affects the cell body itself or results in extensive degeneration of a distal axon.

Mammalian neural pathways also are at risk due to damage caused by neoplastic lesions. Neoplasias of both the neurons and glial cells have been identified. Transformed cells of neural origin generally lose their ability to behave as normal differentiated cells and can destroy neural pathways by loss of function. In addition, the proliferating tumors may induce lesions by distorting normal nerve tissue structure, inhibiting pathways by compressing nerves, inhibiting cerebrospinal

fluid or blood supply flow, and/or by stimulating the body's immune response. Metastatic tumors, which are a significant cause of neoplastic lesions in the brain and spinal cord, also similarly may damage neural pathways and induce neuronal cell death.

One type of morphoregulatory molecule associated with neuronal cell growth, differentiation and development is the cell adhesion molecule ("CAM"), most notably the nerve cell adhesion molecule (N-CAM). CAMs belong to the immunoglobulin super-family and mediate cell-cell interactions in developing and adult tissues through homophilic binding, i.e., CAM-CAM binding on apposing cells. A number of different CAMs currently have been identified. Of these, the most thoroughly studied to date are N-CAM and L-CAM (liver cell adhesion molecules), both of which have been identified on all cells at early stages of development, as well as in different adult tissues. In neural tissue development, N-CAM expression is believed to be important in tissue organization, neuronal migration, nerve-muscle tissue adhesion, retinal formation, synaptogenesis, and neural degeneration. Reduced N-CAM expression also is thought to be associated with nerve dysfunction. For example, expression of at least one form of N-CAM, N-CAM-180, is reduced in a mouse dysmyelinating mutant (Bhat (1988) Brain Res. 452:373-377). Reduced levels of N-CAM also have been associated with normal pressure hydrocephalus (Werdelin (1989) Acta Neurol. Scand. 79:177-181), and with type II schizophrenia (Lyons et al., (1988) Biol. Psychiatry 23:769-775.) In addition, antibodies to N-CAM have been shown to disrupt functional recovery in injured nerves (Remsen (1990) Exp. Neurobiol. 110:268-273).

It is an object of this invention to provide methods for enhancing survival of neurons at risk of dying in a mammal. Another object is to provide methods for maintaining neural pathways in vivo at risk of injury, or following damage to nerve tissue due to mechanical or chemical trauma, a neuropathy, or a neoplastic lesion. Another object is to provide compositions and devices for repairing gaps in a neural pathway of the peripheral nervous system. Yet another object is to provide a means for redifferentiating transformed cells defining neural pathways, particularly transformed cells of neural origin. Another object is to provide a means for stimulating CAM expression, particularly N-CAM expression in a cell. Yet another object is to provide methods for monitoring the status of nerve tissue by monitoring fluctuations in protein levels present in nerve tissue, serum and/or cerebrospinal fluid. These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

08937756-09297
465260-952680

Summary of the Invention

5 The present invention provides methods and
compositions for maintaining neural pathways in a
mammal in vivo, including methods for enhancing the
survival of neural cells.

10 In one aspect, the invention features compositions
and therapeutic treatment methods that comprise the
step of administering to a mammal a therapeutically
effective amount of a morphogenic protein
("morphogen"), as defined herein, upon injury to a
neural pathway, or in anticipation of such injury, for
15 a time and at a concentration sufficient to maintain
the neural pathway, including repairing damaged
pathways, or inhibiting additional damage thereto.

20 In another aspect, the invention features
compositions and therapeutic treatment methods for
maintaining neural pathways in a mammal in vivo which
include administering to the mammal, upon injury to a
neural pathway or in anticipation of such injury, a
compound that stimulates in vivo a therapeutically
25 effective concentration of an endogenous morphogen
within the body of the mammal sufficient to maintain
the neural pathway, including repairing damaged
pathways or inhibiting additional damage thereto.
These compounds are referred to herein as morphogen-
30 stimulating agents, and are understood to include
substances which, when administered to a mammal, act on

0893756-09259

tissue(s) or organ(s) that normally are responsible for, or capable of, producing a morphogen and/or secreting a morphogen, and which cause the endogenous level of the morphogen to be altered. The agent may
5 act, for example, by stimulating expression and/or secretion of an endogenous morphogen.

In particular, the invention provides methods for enhancing the survival of neurons at risk of dying,
10 including protecting neurons from the tissue destructive effects associated with the body's immune/inflammatory response to a nerve injury. The invention also provides methods for stimulating neurons to maintain their differentiated phenotype, including
15 inducing the redifferentiation of transformed cells of neuronal origin to a morphology characteristic of untransformed neurons. In one embodiment, the invention provides means for stimulating production of cell adhesion molecules in cells, particularly nerve
20 cell adhesion molecules (N-CAM) in neurons. The invention also provides methods, compositions and devices for stimulating cellular repair of damaged neurons and neural pathways, including regenerating damaged axons of the peripheral and central nervous
25 systems. In addition, the invention also provides means for evaluating the status of nerve tissue, and for detecting and monitoring neuropathies in a mammal by monitoring fluctuations in the morphogen levels or endogenous morphogen antibody levels present in a
30 mammal's serum or cerebrospinal fluid.

As used herein, a "neural pathway" describes a nerve circuit for the passage of electric signals from a source to a target cell site. The pathway includes
35 the neurons through which the electric impulse is

0893756 "093597
465360 9542680

5

In one aspect of the invention, the morphogens described herein are useful in repairing damaged neural pathways of the peripheral nervous system. In particular, the morphogens are useful for repairing damaged pathways, including transected or otherwise damaged nerve fibers (nerves) requiring regeneration of neuronal processes, particularly axons, over extended distances to bridge a gap in the nerve itself, or between the nerve and a post-synaptic cell. Specifically, the morphogens described herein are capable of stimulating complete axonal nerve regeneration, including vascularization and reformation of the protective myelin sheath. The morphogen preferably is provided to the site of injury dispersed in a biocompatible, bioresorbable carrier material capable of maintaining the morphogen at the site and, where necessary, means for directing axonal growth from the proximal to the distal ends of a severed neuron or nerve. For example, means for directing axonal growth may be required where nerve regeneration is to be induced over an extended distance, such as greater than 10 mm. Many carriers capable of providing these functions are envisioned. For example, useful carriers include substantially insoluble materials or viscous solutions prepared as disclosed herein comprising laminin, hyaluronic acid or collagen, or other suitable synthetic, biocompatible polymeric materials such as

polylactic, polyglycolic or polybutyric acids and/or copolymers thereof. The currently preferred carrier comprises an extracellular matrix composition, such as one described herein derived, for example, from mouse
5 sarcoma cells. Also envisioned as especially useful are brain tissue-derived extracellular matrices.

In a particularly preferred embodiment, the morphogen is provided to the site as part of a device
10 wherein the morphogen is disposed in a nerve guidance channel which spans the distance of the damaged pathway. The channel acts both as a protective covering and a physical means for guiding growth of a neuronal process such as an axon. Useful channels
15 comprise a biocompatible membrane or casing, which may be tubular in structure, having a dimension sufficient to span the gap or break in the nerve to be repaired, and having openings adapted to receive severed nerve ends. The casing or membrane may be made of any
20 biocompatible, nonirritating material, such as silicone or a biocompatible polymer such as polyethylene or polyethylene vinyl acetate. The casing also may be composed of biocompatible, bioresorbable polymers, including, for example, collagen, hyaluronic acid,
25 polylactic, polybutyric and polyglycolic acids. In a currently preferred embodiment, the outer surface of the channel is substantially impermeable.

The morphogen may be disposed in the channel in
30 association with a biocompatible carrier material, or it may be adsorbed to or otherwise associated with the inner surface of the casing, such as is described in U.S. Pat. No. 5,011,486, provided that the morphogen is accessible to the severed nerve ends. Additionally,
35 although the nerve guidance channels described herein

generally are tubular in shape, it should be evident to those skilled in the art that various alternative shapes may be employed. The lumen of the guidance channels may, for example, be oval or even square in cross section. Moreover the guidance channels may be constructed of two or more parts which may be clamped together to secure the nerve stumps. Nerve endings may be secured to the nerve guidance channels by means of sutures, biocompatible adhesives such as fibrin glue, or other means known in the medical art.

The morphogens described herein also are envisioned to be useful in autologous peripheral nerve segment implants to bypass damaged neural pathways in the central nervous system, such as in the repair of damaged or detached retinas, or other damage to the optic nerve. Here the morphogen is provided to the site of attachment to stimulate axonal growth at the graft site, particularly where the damaged axonal segment to be bypassed occurs far from the neuronal cell body.

The morphogens described herein also are useful for enhancing survival of neuronal cells at risk of dying, thereby preventing, limiting or otherwise inhibiting damage to neural pathways. Non-mitotic neurons are at risk of dying as a result of a neuropathy or other cellular dysfunction of a neuron or glial cell inducing cell death, or following a chemical or mechanical lesion to the cell or its surrounding tissue. The chemical lesions may result from known toxic agents, including lead, ethanol, ammonia, formaldehyde and many other organic solvents, as well as the toxins in cigarette smoke and opiates. Excitatory amino acids, such as glutamate also may play a role in the

pathogenesis of neuronal cell death (see Freese et al. (1990) Brain Res. 521:254-264). Neuronal cell death also is thought to be a significant contributing factor in a number of neurodegenerative diseases, including

5 Alzheimer's disease, Huntington's chorea, and Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis. The etiology of these neuropathies may be metabolic, as results in hepatic encephalopathy, infectious, toxic, autoimmune, nutritional or ischemic.

10 In addition, ethanol and a number of other toxins also have been identified as significant contributing factors in neurodegenerative diseases. The morphogens described herein may be provided to cells at risk of dying to enhance their survival and thereby protect the

15 integrity of the neural pathway. The morphogens may be provided directly to the site, or they may be provided systemically. Alternatively, as described above, an agent capable of stimulating endogenous morphogen expression and/or secretion, preferably in cells

20 associated with the nerve tissue of interest, may be administered to the mammal.

In another aspect of the invention, the method disclosed is useful for redifferentiating transformed

25 cells, particularly transformed cells of neuronal or glial origin, such that the morphogen-treated cells are induced to display a morphology characteristic of untransformed cells. Where the transformed cells are cells of neuronal origin, morphogen treatment

30 preferably induces cell rounding and cell aggregation (clumping), cell-cell adhesion, neurite outgrowth formation and elongation, and N-CAM production. The methods described herein are anticipated to substantially inhibit or reduce neural cell tumor

35 formation and/or proliferation in nerve tissue. It is

003756-0259
465360-9542680

anticipated that the methods of this invention will be useful in substantially reducing the effects of various carcinomas of nerve tissue origin such as retinoblastomas, neuroblastomas, and gliomas or glioblastomas. In addition, the method also is anticipated to aid in inhibiting neoplastic lesions caused by metastatic tissue. Metastatic tumors are one of the most common neoplasms of the CNS, as they can reach the intracranial compartment through the bloodstream. Metastatic tumors may damage neural pathways for example, by distorting normal nerve tissue structure, compressing nerves, blocking flow of cerebrospinal fluid or the blood supply nourishing brain tissue, and/or by stimulating the body's immune response.

In another aspect of the invention, the morphogens described herein are useful for providing neuroprotective effects to alleviate neural pathway damage associated with the body's immune/inflammatory response to an initial injury to nerve tissue. Such a response may follow trauma to nerve tissue, caused, for example, by an autoimmune dysfunction, neoplastic lesion, infection, chemical or mechanical trauma, disease, by interruption of blood flow to the neurons or glial cells, for example following ischemia or hypoxia, or by other trauma to the nerve or surrounding material. For example, the primary damage resulting from hypoxia or ischemia-reperfusion following occlusion of a neural blood supply, as in an embolic stroke, is believed to be immunologically associated. In addition, at least part of the damage associated with a number of primary brain tumors also appears to be immunologically related. Application of the morphogen directly to the cells to be treated, or

265260-9524680

providing the morphogen to the mammal systemically, for example, intravenously or indirectly by oral administration, may be used to alleviate and/or inhibit the immunologically related response to a neural injury. Alternatively, administration of an agent capable of stimulating morphogen expression and/or secretion in vivo, preferably at the site of injury, also may be used. Where the injury is to be induced, as during surgery or other aggressive clinical treatment, the morphogen or agent may be provided prior to induction of the injury to provide a neuroprotective effect to the nerve tissue at risk.

In still another aspect, the invention described herein provides methods for supporting the growth and maintenance of differentiated neurons, including inducing neurons to continue expressing their phenotype. It is anticipated that this activity will be particularly useful in the treatment of nerve tissue disorders where loss of function is caused by reduced or lost cellular metabolic function and cells become senescent or quiescent, such as is thought to occur in aging cells and to be manifested in Alzheimer's disease. Application of the morphogen directly to cells to be treated, or providing it systemically by parenteral or oral administration stimulates these cells to continue expressing their phenotype, significantly inhibiting and/or reversing the effects of the cellular metabolic dysfunction, thereby maintaining the neural pathway at risk. Alternatively, administration of an agent capable of stimulating endogenous morphogen expression and/or secretion in vivo may be used.

0893756-092597
765260-954680

In still another aspect, the invention provides methods for stimulating CAM expression levels in a cell, particularly N-CAM expression in neurons. CAMs are molecules defined as carrying out cell-cell
5 interactions necessary for tissue formation. CAMs are believed to play a fundamental regulatory role in tissue development, including tissue boundary formation, embryonic induction and migration, and tissue stabilization and regeneration. Altered CAM
10 levels have been implicated in a number of tissue disorders, including congenital defects, neoplasias, and degenerative diseases.

In particular, N-CAM expression is associated with
15 normal neuronal cell development and differentiation, including retinal formation, synaptogenesis, and nerve-muscle tissue adhesion. Inhibition of one or more of the N-CAM isoforms is known to prevent proper tissue development. Altered N-CAM expression levels also are
20 associated with neoplasias, including neuroblastomas (see infra), as well as with a number of neuropathies, including normal pressure hydrocephalous and type II schizophrenia. Application of the morphogen directly to the cells to be treated, or providing the morphogen
25 to the mammal systemically, for example, parenterally, or indirectly by oral administration, may be used to induce cellular expression of one or more CAMs, particularly N-CAMs. Alternatively, administration of an agent capable of stimulating morphogen expression
30 and/or secretion in vivo, preferably at the site of injury, also may be used to induce CAM production.

CAMs also have been postulated as part of a morphoregulatory pathway whose activity is induced by a
35 to date unidentified molecule (See, for example,

0893756-09597
465260-952680

Edelman, G.M. (1986) Ann. Rev. Cell Biol. 2:81-116). Without being limited to any given theory, the morphogens described herein may act as the inducer of this pathway.

5

Finally, modulations of endogenous morphogen levels may be monitored as part of a method of detecting nerve tissue dysfunction. Specifically, modulations in endogenous morphogen levels are anticipated to reflect changes in nerve tissue status. Morphogen expression may be monitored directly in biopsied cell samples, in cerebrospinal fluid, or serum. Alternatively, morphogen levels may be assessed by detecting changes in the levels of endogenous antibodies to the morphogen. For example, one may obtain serum samples from a mammal, and then detect the concentration of morphogen or antibody present in the fluid by standard protein detection means known to those skilled in the art. As an example, binding protein capable of interacting specifically with the morphogen of interest such as an anti-morphogen antibody may be used to detect a morphogen in a standard immunoassay. The morphogen levels detected then may be compared to a previously determined standard or reference level, with changes in the detected levels being indicative of the status of the tissue.

In one preferred embodiment of the invention, the morphogen or morphogen-stimulating agent is administered systemically to the individual, e.g., orally or parenterally. In another embodiment of the invention, the morphogen may be provided directly to the nerve tissue, e.g., by injection to the cerebral spinal fluid or to a nerve tissue locus.

35

089377WE-092297
265260-9524689

In any treatment method of the invention, "administration of morphogen" refers to the administration of the morphogen, either alone or in combination with other molecules. For example, the
5 mature form of the morphogen may be provided in association with its precursor "pro" domain, which is known to enhance the solubility of the protein. Other useful molecules known to enhance protein solubility include casein and other milk components, as well as
10 various serum proteins. Additional useful molecules which may be associated with the morphogen or morphogen-stimulating agent include tissue targeting molecules capable of directing the morphogen or morphogen-stimulating agent to nerve tissue. Tissue
15 targeting molecules envisioned to be useful in the treatment protocols of this invention include antibodies, antibody fragments or other binding proteins which interact specifically with surface molecules on nerve tissue cells.

20

Still another useful tissue targeting molecule is part or all of the morphogen precursor "pro" domain, particularly that of OP-1 or GDF-1. These proteins are found naturally associated with nerve tissue but also
25 may be synthesized in other tissues and targeted to nerve tissue after secretion from the synthesizing tissue. For example, while the protein has been shown to be active in bone tissue, the primary source of OP-1 synthesis appears to be the tissue of the urogenic
30 system (e.g., renal and bladder tissue), with secondary expression levels occurring in the brain, heart and lungs (see below.) Moreover, the protein has been

identified in serum, saliva and various milk forms. In addition, the secreted form of the protein comprises the mature dimer in association with the pro domain of the intact morphogen sequence. Accordingly, the associated morphogen pro domains may act to target specific morphogens to different tissues in vivo.

Associated tissue targeting or solubility-enhancing molecules also may be covalently linked to the morphogen using standard chemical means, including acid-labile linkages, which likely will be preferentially cleaved in the acidic environment of bone remodeling sites.

Finally, the morphogens or morphogen-stimulating agents provided herein also may be administered in combination with other molecules known to be beneficial in maintaining neural pathways, including, for example, nerve growth factors and anti-inflammatory agents.

Where the morphogen is intended for use as a therapeutic for disorders of the CNS, an additional problem must be addressed: overcoming the so-called "blood-brain barrier", the brain capillary wall structure that effectively screens out all but selected categories of molecules present in the blood, preventing their passage into the brain. The blood-brain barrier may be bypassed effectively by direct infusion of the morphogen or morphogen-stimulating agent into the brain. Alternatively, the morphogen or morphogen-stimulating agent may be modified to enhance its transport across the blood-brain barrier. For example, truncated forms of the morphogen or a morphogen-stimulating agent may be most successful. Alternatively, the morphogen or

morphogen-stimulating agent may be modified to render it more lipophilic, or it may be conjugated to another molecule which is naturally transported across the barrier, using standard means known to those skilled in the art, as, for example, described in Pardridge, Endocrine Reviews 7:314-330 (1986) and U.S. Pat. No. 4,801,575.

Accordingly, as used herein, a functional "analog" of a morphogen refers to a protein having morphogenic biological activity but possessing additional structural differences compared to a morphogen as defined herein, e.g., having additional chemical moieties not normally a part of a morphogen. Such moieties (introduced, for example, by acylation, alkylation, cationization, or glycosylation reactions, or other means for conjugating the moiety to the morphogen) may improve the molecule's solubility, absorption, biological half-life, or transport, e.g., across the blood-brain barrier.

Among the morphogens useful in this invention are proteins originally identified as osteogenic proteins, such as the OP-1, OP-2 and CBMP2 proteins, as well as amino acid sequence-related proteins such as DPP (from *Drosophila*), Vgl (from *Xenopus*), Vgr-1 (from mouse, see U.S. 5,011,691 to Oppermann et al.), GDF-1 (from mouse, see Lee (1991) PNAS 88:4250-4254), all of which are presented in Table II and Seq. ID Nos.5-14), and the recently identified 60A protein (from *Drosophila*, Seq. ID No. 24, see Wharton et al. (1991) PNAS 88:9214-9218.) The members of this family, which include members of the TGF- β super-family of proteins, share substantial amino acid sequence homology in their C-terminal regions. The proteins are translated as a

precursor, having an N-terminal signal peptide sequence, typically less than about 30 residues, followed by a "pro" domain that is cleaved to yield the mature sequence. The signal peptide is cleaved rapidly upon translation, at a cleavage site that can be predicted in a given sequence using the method of Von Heijne ((1986) Nucleic Acids Research 14:4683-4691.) Table I, below, describes the various morphogens identified to date, including their nomenclature as used herein, their Seq. ID references, and publication sources for the amino acid sequences for the full length proteins not included in the Seq. Listing. The disclosure of these publications is incorporated herein by reference.

TABLE I

"OP-1"	Refers generically to the group of morphogenically active proteins expressed from part or all of a DNA sequence encoding OP-1 protein, including allelic and species variants thereof, e.g., human OP-1 ("hOP-1", Seq. ID No. 5, mature protein amino acid sequence), or mouse OP-1 ("mOP-1", Seq. ID No. 6, mature protein amino acid sequence.) The conserved seven cysteine skeleton is defined by residues 38 to 139 of Seq. ID Nos. 5 and 6. The cDNA sequences and the amino acids encoding the full length proteins are provided in Seq. ID Nos. 16 and 17 (hOP1) and Seq. ID Nos. 18 and 19 (mOP1.) The mature proteins are defined
--------	---

by residues 293-431 (hOP1) and 292-430 (mOP1). The "pro" regions of the proteins, cleaved to yield the mature, morphogenically active proteins are defined essentially by residues 30-292 (hOP1) and residues 30-291 (mOP1).

"OP-2" refers generically to the group of active proteins expressed from part or all of a DNA sequence encoding OP-2 protein, including allelic and species variants thereof, e.g., human OP-2 ("hOP-2", Seq. ID No. 7, mature protein amino acid sequence) or mouse OP-2 ("mOP-2", Seq. ID No. 8, mature protein amino acid sequence). The conserved seven cysteine skeleton is defined by residues 38 to 139 of Seq. ID Nos. 7 and 8. The cDNA sequences and the amino acids encoding the full length proteins are provided in Seq. ID Nos. 20 and 21 (hOP2) and Seq. ID Nos. 22 and 23 (mOP2.) The mature proteins are defined essentially by residues 264-402 (hOP2) and 261-399 (mOP2). The "pro" regions of the proteins, cleaved to yield the mature, morphogenically active proteins likely are defined essentially by residues 18-263 (hOP2) and residues 18-260 (mOP2). (Another cleavage site also occurs 21 residues upstream for both OP-2 proteins.)

- 0893756-09999
465260" 9522680
- 5 "CBMP2" refers generically to the morphogenically active proteins expressed from a DNA sequence encoding the CBMP2 proteins, including allelic and species variants thereof, e.g., human CBMP2A ("CBMP2A(fx)", Seq ID No. 9) or human CBMP2B DNA ("CBMP2B(fx)", Seq. ID No. 10). The amino acid sequence for the full length proteins, referred to in the literature as BMP2A and BMP2B, or BMP2 and BMP4, appear in Wozney, et al. (1988) Science 242:1528-1534. The pro domain for BMP2 (BMP2A) likely includes residues 25-248 or 25-282; the mature protein, residues 249-396 or 283-396. The pro domain for BMP4 (BMP2B) likely includes residues 25-256 or 25-292; the mature protein, residues 257-408 or 293-408.
- 20 "DPP(fx)" refers to protein sequences encoded by the *Drosophila* DPP gene and defining the conserved seven cysteine skeleton (Seq. ID No. 11). The amino acid sequence for the full length protein appears in Padgett, et al (1987) Nature 325: 81-84. The pro domain likely extends from the signal peptide cleavage site to residue 456; the mature protein likely is defined by residues 457-588.
- 30 "Vgl(fx)" refers to protein sequences encoded by the *Xenopus* Vgl gene and defining the conserved seven cysteine skeleton (Seq. ID No. 12). The amino acid sequence for the full length protein appears in
- 35

Weeks (1987) Cell 51: 861-867. The prodomain likely extends from the signal peptide cleavage site to residue 246; the mature protein likely is defined by residues 247-360.

5

"Vgr-1(fx)" refers to protein sequences encoded by the murine Vgr-1 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 13). The amino acid sequence for the full length protein appears in Lyons, et al, (1989) PNAS 86: 4554-4558. The prodomain likely extends from the signal peptide cleavage site to residue 299; the mature protein likely is defined by residues 300-438.

10

15

"GDF-1(fx)" refers to protein sequences encoded by the human GDF-1 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 14). The cDNA and encoded amino sequence for the full length protein is provided in Seq. ID. No. 32. The prodomain likely extends from the signal peptide cleavage site to residue 214; the mature protein likely is defined by residues 215-372.

20

25

"60A" refers generically to the morphogenically active proteins expressed from part or all of a DNA sequence (from the Drosophila 60A gene) encoding the 60A proteins (see Seq. ID No. 24 wherein the cDNA and encoded amino acid sequence for the full length protein is provided). "60A(fx)" refers to

30

35

265260" 9542630

the protein sequences defining the conserved seven cysteine skeleton (residues 354 to 455 of Seq. ID No. 24.) The prodomain likely extends from the signal peptide cleavage site to residue 324; the mature protein likely is defined by residues 325-455.

- 5
- 10 "BMP3(fx)" refers to protein sequences encoded by the human BMP3 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 26). The amino acid sequence for the full length protein appears in Wozney et al. (1988) Science 242: 1528-1534. The pro domain likely extends from the signal peptide cleavage site to residue 290; the mature protein likely is defined by residues 291-472.
- 15
- 20 "BMP5(fx)" refers to protein sequences encoded by the human BMP5 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 27). The amino acid sequence for the full length protein appears in Celeste, et al. (1991) PNAS 87: 9843-9847. The pro domain likely extends from the signal peptide cleavage site to residue 316; the mature protein likely is defined by residues 317-454.
- 25
- 30 "BMP6(fx)" refers to protein sequences encoded by the human BMP6 gene and defining the conserved seven cysteine skeleton (Seq. ID No. 28). The amino acid sequence for the full length protein appear sin Celeste, et al.
- 35

0893756-09597
269260-952680

5

10

20

30

35

acting as a morphogen as defined herein. Specifically,
the morphogens generally are capable of all of the
following biological functions in a morphogenically
permissive environment: stimulating proliferation of
5 progenitor cells; stimulating the differentiation of
progenitor cells; stimulating the proliferation of
differentiated cells; and supporting the growth and
maintenance of differentiated cells, including the
"redifferentiation" of transformed cells. In addition,
10 it is also anticipated that these morphogens are
capable of inducing redifferentiation of committed
cells under appropriate environmental conditions.

In one preferred aspect, the morphogens of
15 this invention comprise one of two species of generic
amino acid sequences: Generic Sequence 1 (Seq. ID
No. 1) or Generic Sequence 2 (Seq. ID No. 2); where
each Xaa indicates one of the 20 naturally-occurring
L-isomer, α -amino acids or a derivative thereof.
20 Generic Sequence 1 comprises the conserved six cysteine
skeleton and Generic Sequence 2 comprises the conserved
six cysteine skeleton plus the additional cysteine
identified in OP-2 (see residue 36, Seq. ID No. 2). In
another preferred aspect, these sequences further
25 comprise the following additional sequence at their N-
terminus:

Cys Xaa Xaa Xaa Xaa (Seq. ID No. 15)
1 5

30

Preferred amino acid sequences within the
foregoing generic sequences include: Generic Sequence
3 (Seq. ID No. 3), Generic Sequence 4 (Seq. ID No. 4),
Generic Sequence 5 (Seq. ID No. 30) and Generic
35 Sequence 6 (Seq. ID No. 31), listed below. These

08937756-092597

```

25      Generic Sequence 3
        Leu Tyr Val Xaa Phe
          1                5
        Xaa Xaa Xaa Gly Trp Xaa Xaa Trp Xaa
              10
30      Xaa Ala Pro Xaa Gly Xaa Xaa Ala
          15                20

```

Leu Tyr Val Xaa Phe

1 5

Xaa Xaa Xaa Gly Trp Xaa Xaa Trp Xaa

10

Xaa Ala Pro Xaa Gly Xaa Xaa Ala

15 20

Xaa Tyr Cys Xaa Gly Xaa Cys Xaa
 25 30
 Xaa Pro Xaa Xaa Xaa Xaa Xaa
 35
 5 Xaa Xaa Xaa Asn His Ala Xaa Xaa
 40 45
 Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa
 50
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
 10 55 60
 Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa
 65
 Xaa Xaa Xaa Leu Xaa Xaa Xaa
 70 75
 15 Xaa Xaa Xaa Xaa Val Xaa Leu Xaa
 80
 Xaa Xaa Xaa Xaa Met Xaa Val Xaa
 85 90
 Xaa Cys Gly Cys Xaa
 20 95

wherein each Xaa is independently selected from a group
 of one or more specified amino acids defined as
 follows: "Res." means "residue" and Xaa at res.4 =
 (Ser, Asp or Glu); Xaa at res.6 = (Arg, Gln, Ser or
 25 Lys); Xaa at res.7 = (Asp or Glu); Xaa at res.8 = (Leu
 or Val); Xaa at res.11 = (Gln, Leu, Asp, His or Asn);
 Xaa at res.12 = (Asp, Arg or Asn); Xaa at res.14 = (Ile
 or Val); Xaa at res.15 = (Ile or Val); Xaa at res.18 =

0093756-09259
265260-9546680

(Glu, Gln, Leu, Lys, Pro or Arg); Xaa at res.20 = (Tyr or Phe); Xaa at res.21 = (Ala, Ser, Asp, Met, His, Leu or Gln); Xaa at res.23 = (Tyr, Asn or Phe); Xaa at res.26 = (Glu, His, Tyr, Asp or Gln); Xaa at res.28 = (Glu, Lys, Asp or Gln); Xaa at res.30 = (Ala, Ser, Pro or Gln); Xaa at res.31 = (Phe, Leu or Tyr); Xaa at res.33 = (Leu or Val); Xaa at res.34 = (Asn, Asp, Ala or Thr); Xaa at res.35 = (Ser, Asp, Glu, Leu or Ala); Xaa at res.36 = (Tyr, Cys, His, Ser or Ile); Xaa at res.37 = (Met, Phe, Gly or Leu); Xaa at res.38 = (Asn or Ser); Xaa at res.39 = (Ala, Ser or Gly); Xaa at res.40 = (Thr, Leu or Ser); Xaa at res.44 = (Ile or Val); Xaa at res.45 = (Val or Leu); Xaa at res.46 = (Gln or Arg); Xaa at res.47 = (Thr, Ala or Ser); Xaa at res.49 = (Val or Met); Xaa at res.50 = (His or Asn); Xaa at res.51 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa at res.52 = (Ile, Met, Asn, Ala or Val); Xaa at res.53 = (Asn, Lys, Ala or Glu); Xaa at res.54 = (Pro or Ser); Xaa at res.55 = (Glu, Asp, Asn, or Gly); Xaa at res.56 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser or Ala); Xaa at res.57 = (Val, Ala or Ile); Xaa at res.58 = (Pro or Asp); Xaa at res.59 = (Lys or Leu); Xaa at res.60 = (Pro or Ala); Xaa at res.63 = (Ala or Val); Xaa at res.65 = (Thr or Ala); Xaa at res.66 = (Gln, Lys, Arg or Glu); Xaa at res.67 = (Leu, Met or Val); Xaa at res.68 = (Asn, Ser or Asp); Xaa at res.69 = (Ala, Pro or Ser); Xaa at res.70 = (Ile, Thr or Val); Xaa at res.71 = (Ser or Ala); Xaa at res.72 = (Val or Met); Xaa at res.74 = (Tyr or Phe); Xaa at res.75 = (Phe, Tyr or Leu); Xaa at res.76 = (Asp or Asn); Xaa at res.77 = (Asp, Glu, Asn or Ser); Xaa at res.78 = (Ser, Gln, Asn or Tyr); Xaa at res.79 = (Ser, Asn, Asp or Glu); Xaa at res.80 = (Asn, Thr or Lys); Xaa at res.82 = (Ile or

00937756-092597

Val); Xaa at res.84 = (Lys or Arg); Xaa at res.85 =
(Lys, Asn, Gln or His); Xaa at res.86 = (Tyr or His);
Xaa at res.87 = (Arg, Gln or Glu); Xaa at res.88 =
(Asn, Glu or Asp); Xaa at res.90 = (Val, Thr or Ala);
5 Xaa at res.92 = (Arg, Lys, Val, Asp or Glu); Xaa at
res.93 = (Ala, Gly or Glu); and Xaa at res.97 = (His or
Arg);

Generic Sequence 4

10 Cys Xaa Xaa Xaa Xaa Leu Tyr Val Xaa Phe
1 5 10
Xaa Xaa Xaa Gly Trp Xaa Xaa Trp Xaa
15
Xaa Ala Pro Xaa Gly Xaa Xaa Ala
20 25
Xaa Tyr Cys Xaa Gly Xaa Cys Xaa
30 35
Xaa Pro Xaa Xaa Xaa Xaa Xaa
20 40
Xaa Xaa Xaa Asn His Ala Xaa Xaa
45 50
Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa
55
25 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
60 65
Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa
70
Xaa Xaa Xaa Leu Xaa Xaa Xaa
30 75 80
Xaa Xaa Xaa Xaa Val Xaa Leu Xaa
85
Xaa Xaa Xaa Xaa Met Xaa Val Xaa
90 95
35 Xaa Cys Gly Cys Xaa
100

0037756-0959
265260-9572680

wherein each Xaa is independently selected from a group of one or more specified amino acids as defined by the following: "Res." means "residue" and Xaa at res.2 = (Lys or Arg); Xaa at res.3 = (Lys or Arg); Xaa at res.4 = (His or Arg); Xaa at res.5 = (Glu, Ser, His, Gly, Arg or Pro); Xaa at res.9 = (Ser, Asp or Glu); Xaa at res.11 = (Arg, Gln, Ser or Lys); Xaa at res.12 = (Asp or Glu); Xaa at res.13 = (Leu or Val); Xaa at res.16 = (Gln, Leu, Asp, His or Asn); Xaa at res.17 = (Asp, Arg, or Asn); Xaa at res.19 = (Ile or Val); Xaa at res.20 = (Ile or Val); Xaa at res.23 = (Glu, Gln, Leu, Lys, Pro or Arg); Xaa at res.25 = (Tyr or Phe); Xaa at res.26 = (Ala, Ser, Asp, Met, His, Leu, or Gln); Xaa at res.28 = (Tyr, Asn or Phe); Xaa at res.31 = (Glu, His, Tyr, Asp or Gln); Xaa at res.33 = Glu, Lys, Asp or Gln; Xaa at res.35 = (Ala, Ser or Pro); Xaa at res.36 = (Phe, Leu or Tyr); Xaa at res.38 = (Leu or Val); Xaa at res.39 = (Asn, Asp, Ala or Thr); Xaa at res.40 = (Ser, Asp, Glu, Leu or Ala); Xaa at res.41 = (Tyr, Cys, His, Ser or Ile); Xaa at res.42 = (Met, Phe, Gly or Leu); Xaa at res.44 = (Ala, Ser or Gly); Xaa at res.45 = (Thr, Leu or Ser); Xaa at res.49 = (Ile or Val); Xaa at res.50 = (Val or Leu); Xaa at res.51 = (Gln or Arg); Xaa at res.52 = (Thr, Ala or Ser); Xaa at res.54 = (Val or Met); Xaa at res.55 = (His or Asn); Xaa at res.56 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa at res.57 = (Ile, Met, Asn, Ala or Val); Xaa at res.58 = (Asn, Lys, Ala or Glu); Xaa at res.59 = (Pro or Ser); Xaa at res.60 = (Glu, Asp, or Gly); Xaa at res.61 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser or Ala); Xaa at res.62 = (Val, Ala or Ile); Xaa at res.63 = (Pro or Asp); Xaa at res.64 = (Lys or Leu); Xaa at res.65 = (Pro or Ala); Xaa at res.68 = (Ala or Val); Xaa at res.70 = (Thr or Ala); Xaa at res.71 = (Gln, Lys, Arg or Glu); Xaa at res.72 = (Leu, Met or Val); Xaa at res.73 = (Asn, Ser or Asp);

0893755-09597
265260-9544880

15

25

30

35

5

Generic Sequence 5

15

Leu Xaa Xaa Xaa Phe

5

Xaa Xaa Xaa Gly Trp Xaa Xaa Trp Xaa

10

Xaa Xaa Pro Xaa Xaa Xaa Xaa Ala

20

Xaa Tyr Cys Xaa Gly Xaa Cys Xaa

30

Xaa Pro Xaa Xaa Xaa Xaa Xaa

25

35

Xaa Xaa Xaa Asn His Ala Xaa Xaa

45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa

50

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys

60

Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa

65

70

Xaa Xaa Xaa Xaa Val Xaa Leu Xaa

5 Xaa Xaa Xaa Xaa Met Xaa Val Xaa

85

90

Xaa Cys Xaa Cys Xaa

95

wherein each Xaa is independently selected from a group of one or more specified amino acids defined as follows: "Res." means "residue" and Xaa at res.2 = (Tyr or Lys); Xaa at res.3 = Val or Ile); Xaa at res.4 = (Ser, Asp or Glu); Xaa at res.6 = (Arg, Gln, Ser, Lys or Ala); Xaa at res.7 = (Asp, Glu or Lys); Xaa at res.8 = (Leu, Val or Ile); Xaa at res.11 = (Gln, Leu, Asp, His, Asn or Ser); Xaa at res.12 = (Asp, Arg, Asn or Glu); Xaa at res.14 = (Ile or Val); Xaa at res.15 = (Ile or Val); Xaa at res.16 (Ala or Ser); Xaa at res.18 = (Glu, Gln, Leu, Lys, Pro or Arg); Xaa at res.19 = (Gly or Ser); Xaa at res.20 = (Tyr or Phe); Xaa at res.21 = (Ala, Ser, Asp, Met, His, Gln, Leu or Gly); Xaa at res.23 = (Tyr, Asn or Phe); Xaa at res.26 = (Glu, His, Tyr, Asp, Gln or Ser); Xaa at res.28 = (Glu, Lys, Asp, Gln or Ala); Xaa at res.30 = (Ala, Ser, Pro, Gln or Asn); Xaa at res.31 = (Phe, Leu or Tyr); Xaa at res.33 = (Leu, Val or Met); Xaa at res.34 = (Asn, Asp, Ala, Thr or Pro); Xaa at res.35 = (Ser, Asp, Glu, Leu, Ala or Lys); Xaa at res.36 = (Tyr, Cys, His, Ser or Ile); Xaa at res.37 = (Met, Phe, Gly or Leu); Xaa at res.38 = (Asn, Ser or Lys); Xaa at res.39 = (Ala, Ser, Gly or Pro); Xaa at res.40 = (Thr, Leu or Ser); Xaa at res.44 = (Ile, Val or Thr); Xaa at res.45 = (Val, Leu

or Ile); Xaa at res.46 = (Gln or Arg); Xaa at res.47 =
(Thr, Ala or Ser); Xaa at res.48 = (Leu or Ile); Xaa at
res.49 = (Val or Met); Xaa at res.50 = (His, Asn or
Arg); Xaa at res.51 = (Phe, Leu, Asn, Ser, Ala or Val);
5 Xaa at res.52 = (Ile, Met, Asn, Ala, Val or Leu); Xaa
at res.53 = (Asn, Lys, Ala, Glu, Gly or Phe); Xaa at
res.54 = (Pro, Ser or Val); Xaa at res.55 = (Glu, Asp,
Asn, Gly, Val or Lys); Xaa at res.56 = (Thr, Ala, Val,
Lys, Asp, Tyr, Ser, Ala, Pro or His); Xaa at res.57 =
10 (Val, Ala or Ile); Xaa at res.58 = (Pro or Asp); Xaa at
res.59 = (Lys, Leu or Glu); Xaa at res.60 = (Pro or
Ala); Xaa at res.63 = (Ala or Val); Xaa at res.65 =
(Thr, Ala or Glu); Xaa at res.66 = (Gln, Lys, Arg or
Glu); Xaa at res.67 = (Leu, Met or Val); Xaa at res.68
15 = (Asn, Ser, Asp or Gly); Xaa at res.69 = (Ala, Pro or
Ser); Xaa at res.70 = (Ile, Thr, Val or Leu); Xaa at
res.71 = (Ser, Ala or Pro); Xaa at res.72 = (Val, Met
or Ile); Xaa at res.74 = (Tyr or Phe); Xaa at res.75 =
(Phe, Tyr, Leu or His); Xaa at res.76 = (Asp, Asn or
20 Leu); Xaa at res.77 = (Asp, Glu, Asn or Ser); Xaa at
res.78 = (Ser, Gln, Asn, Tyr or Asp); Xaa at res.79 =
(Ser, Asn, Asp, Glu or Lys); Xaa at res.80 = (Asn, Thr
or Lys); Xaa at res.82 = (Ile, Val or Asn); Xaa at
res.84 = (Lys or Arg); Xaa at res.85 = (Lys, Asn, Gln,
25 His or Val); Xaa at res.86 = (Tyr or His); Xaa at
res.87 = (Arg, Gln, Glu or Pro); Xaa at res.88 = (Asn,
Glu or Asp); Xaa at res.90 = (Val, Thr, Ala or Ile);
Xaa at res.92 = (Arg, Lys, Val, Asp or Glu); Xaa at
res.93 = (Ala, Gly, Glu or Ser); Xaa at res.95 = (Gly
30 or Ala) and Xaa at res.97 = (His or Arg).

265260" 95426630

Generic Sequence 6

	Cys	Xaa	Xaa	Xaa	Xaa	Leu	Xaa	Xaa	Xaa	Phe
	1					5				10
5	Xaa	Xaa	Xaa	Gly	Trp	Xaa	Xaa	Trp	Xaa	
						15				
	Xaa	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Ala		
	20					25				
	Xaa	Tyr	Cys	Xaa	Gly	Xaa	Cys	Xaa		
10			30					35		
	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa			
						40				
	Xaa	Xaa	Xaa	Asn	His	Ala	Xaa	Xaa		
				45				50		
15	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa		
						55				
	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Cys		
				60				65		
	Cys	Xaa	Pro	Xaa	Xaa	Xaa	Xaa	Xaa		
20				70						
	Xaa	Xaa	Xaa	Leu	Xaa	Xaa	Xaa			
				75			80			
	Xaa	Xaa	Xaa	Xaa	Val	Xaa	Leu	Xaa		
				85						
25	Xaa	Xaa	Xaa	Xaa	Met	Xaa	Val	Xaa		
				90			95			
	Xaa	Cys	Xaa	Cys	Xaa					
				100						

30 wherein each Xaa is independently selected from a group
of one or more specified amino acids as defined by the
following: "Res." means "residue" and Xaa at res.2 =
(Lys, Arg, Ala or Gln); Xaa at res.3 = (Lys, Arg or
Met); Xaa at res.4 = (His, Arg or Gln); Xaa at res.5 =
35 (Glu, Ser, His, Gly, Arg, Pro, Thr, or Tyr); Xaa at

res.7 = (Tyr or Lys); Xaa at res.8 = (Val or Ile); Xaa
at res.9 = (Ser, Asp or Glu); Xaa at res.11 = (Arg,
Gln, Ser, Lys or Ala); Xaa at res.12 = (Asp, Glu, or
Lys); Xaa at res.13 = (Leu, Val or Ile); Xaa at res.16
5 = (Gln, Leu, Asp, His, Asn or Ser); Xaa at res.17 =
(Asp, Arg, Asn or Glu); Xaa at res.19 = (Ile or Val);
Xaa at res.20 = (Ile or Val); Xaa at res.21 = (Ala or
Ser); Xaa at res.23 = (Glu, Gln, Leu, Lys, Pro or Arg);
Xaa at res.24 = (Gly or Ser); Xaa at res.25 = (Tyr or
10 Phe); Xaa at res.26 = (Ala, Ser, Asp, Met, His, Gln,
Leu, or Gly); Xaa at res.28 = (Tyr, Asn or Phe); Xaa at
res.31 = (Glu, His, Tyr, Asp, Gln or Ser); Xaa at
res.33 = Glu, Lys, Asp, Gln or Ala); Xaa at res.35 =
(Ala, Ser, Pro, Gln or Asn); Xaa at res.36 = (Phe, Leu
15 or Tyr); Xaa at res.38 = (Leu, Val or Met); Xaa at
res.39 = (Asn, Asp, Ala, Thr or Pro); Xaa at res.40 =
(Ser, Asp, Glu, Leu, Ala or Lys); Xaa at res.41 = (Tyr,
Cys, His, Ser or Ile); Xaa at res.42 = (Met, Phe, Gly
or Leu); Xaa at res.43 = (Asn, Ser or Lys); Xaa at
20 res.44 = (Ala, Ser, Gly or Pro); Xaa at res.45 = (Thr,
Leu or Ser); Xaa at res.49 = (Ile, Val or Thr); Xaa at
res.50 = (Val, Leu or Ile); Xaa at res.51 = (Gln or
Arg); Xaa at res.52 = (Thr, Ala or Ser); Xaa at res.53
= (Leu or Ile); Xaa at res.54 = (Val or Met); Xaa at
25 res.55 = (His, Asn or Arg); Xaa at res.56 = (Phe, Leu,
Asn, Ser, Ala or Val); Xaa at res.57 = (Ile, Met, Asn,
Ala, Val or Leu); Xaa at res.58 = (Asn, Lys, Ala, Glu,
Gly or Phe); Xaa at res.59 = (Pro, Ser or Val); Xaa at
res.60 = (Glu, Asp, Gly, Val or Lys); Xaa at res.61 =
30 (Thr, Ala, Val, Lys, Asp, Tyr, Ser, Ala, Pro or His);
Xaa at res.62 = (Val, Ala or Ile); Xaa at res.63 = (Pro
or Asp); Xaa at res.64 = (Lys, Leu or Glu); Xaa at
res.65 = (Pro or Ala); Xaa at res.68 = (Ala or Val);
Xaa at res.70 = (Thr, Ala or Glu); Xaa at res.71 =
35 (Gln, Lys, Arg or Glu); Xaa at res.72 = (Leu, Met or

Val); Xaa at res.73 = (Asn, Ser, Asp or Gly); Xaa at
 res.74 = (Ala, Pro or Ser); Xaa at res.75 = (Ile, Thr,
 Val or Leu); Xaa at res.76 = (Ser, Ala or Pro); Xaa at
 res.77 = (Val, Met or Ile); Xaa at res.79 = (Tyr or
 5 Phe); Xaa at res.80 = (Phe, Tyr, Leu or His); Xaa at
 res.81 = (Asp, Asn or Leu); Xaa at res.82 = (Asp, Glu,
 Asn or Ser); Xaa at res.83 = (Ser, Gln, Asn, Tyr or
 Asp); Xaa at res.84 = (Ser, Asn, Asp, Glu or Lys); Xaa
 at res.85 = (Asn, Thr or Lys); Xaa at res.87 = (Ile,
 10 Val or Asn); Xaa at res.89 = (Lys or Arg); Xaa at
 res.90 = (Lys, Asn, Gln, His or Val); Xaa at res.91 =
 (Tyr or His); Xaa at res.92 = (Arg, Gln, Glu or Pro);
 Xaa at res.93 = (Asn, Glu or Asp); Xaa at res.95 =
 (Val, Thr, Ala or Ile); Xaa at res.97 = (Arg, Lys, Val,
 15 Asp or Glu); Xaa at res.98 = (Ala, Gly, Glu or Ser);
 Xaa at res.100 = (Gly or Ala); and Xaa at res.102 =
 (His or Arg).

Particularly useful sequences for use as
 20 morphogens in this invention include the C-terminal
 domains, e.g., the C-terminal 96-102 amino acid
 residues of Vgl, Vgr-1, DPP, OP-1, OP-2, CBMP-2A,
 CBMP-2B, GDF-1 (see Table II, below, and Seq. ID
 Nos. 5-14), as well as proteins comprising the
 25 C-terminal domains of 60A, BMP3, BMP5 and BMP6 (see
 Seq. ID Nos. 24-28), all of which include at least the
 conserved six or seven cysteine skeleton. In addition,
 biosynthetic constructs designed from the generic
 sequences, such as COP-1, 3-5, 7, 16, disclosed in U.S.
 30 Pat. No. 5,011,691, also are useful. Other sequences
 include the inhibins/activin proteins (see, for
 example, U.S. Pat. Nos. 4,968,590 and 5,011,691).
 Accordingly, other useful sequences are those sharing
 at least 70% amino acid sequence homology or
 35 "similarity", and preferably 80% homology or similarity

083756-092597
 265260-952680

with any of the sequences above. These are anticipated to include allelic and species variants and mutants, and biosynthetic muteins, as well as novel members of this morphogenic family of proteins. Particularly

5 envisioned in the family of related proteins are those proteins exhibiting morphogenic activity and wherein the amino acid changes from the preferred sequences include conservative changes, e.g., those as defined by Dayoff et al., Atlas of Protein Sequence and Structure;
10 vol. 5, Suppl. 3, pp. 345-362, (M.O. Dayoff, ed., Nat'l BioMed. Research Fdn., Washington, D.C. 1979). As used herein, potentially useful sequences are aligned with a known morphogen sequence using the method of Needleman et al. ((1970) J.Mol.Biol. 48:443-453) and identities
15 calculated by the Align program (DNASTAR, Inc.). "Homology" or "similarity" as used herein includes allowed conservative changes as defined by Dayoff et al.

20 The currently most preferred protein sequences useful as morphogens in this invention include those having greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the conserved six cysteine skeleton of hOP1
25 (e.g., residues 43-139 of Seq. ID No. 5). These most preferred sequences include both allelic and species variants of the OP-1 and OP-2 proteins, including the Drosophila 60A protein. Accordingly, in another preferred aspect of the invention, useful morphogens
30 include active proteins comprising species of polypeptide chains having the generic amino acid sequence herein referred to as "OPX", which accommodates the homologies between the various identified species of OP1 and OP2 (Seq. ID No. 29).

5 The morphogens useful in the methods, composition
and devices of this invention include proteins
comprising any of the polypeptide chains described
above, whether isolated from naturally-occurring
10 sources, or produced by recombinant DNA or other
synthetic techniques, and includes allelic and species
variants of these proteins, naturally-occurring or
biosynthetic mutants thereof, as well as various
truncated and fusion constructs. Deletion or addition
15 mutants also are envisioned to be active, including
those which may alter the conserved C-terminal cysteine
skeleton, provided that the alteration does not
functionally disrupt the relationship of these
cysteines in the folded structure. Accordingly, such
20 active forms are considered the equivalent of the
specifically described constructs disclosed herein.
The proteins may include forms having varying
glycosylation patterns, varying N-termini, a family of
related proteins having regions of amino acid sequence
25 homology, and active truncated or mutated forms of
native or biosynthetic proteins, produced by expression
of recombinant DNA in host cells.

25 The morphogenic proteins can be expressed from
intact or truncated cDNA or from synthetic DNAs in
procaryotic or eucaryotic host cells, and purified,
cleaved, refolded, and dimerized to form
morphogenically active compositions. Currently
preferred host cells include E. coli or mammalian
30 cells, such as CHO, COS or BSC cells. A detailed
description of the morphogens useful in the methods,

0037756-09297

compositions and devices of this invention is disclosed
in copending US patent application Serial Nos. 752,764,
filed August 30, 1991, and 667,274, filed March 11,
1991, the disclosure of which are incorporated herein
5 by reference.

Thus, in view of this disclosure, skilled genetic
engineers can isolate genes from cDNA or genomic
libraries of various different species which encode
10 appropriate amino acid sequences, or construct DNAs
from oligonucleotides, and then can express them in
various types of host cells, including both procaryotes
and eucaryotes, to produce large quantities of active
proteins capable of maintaining neural pathways in a
15 mammal, including enhancing the survival of neurons at
risk of dying and stimulating nerve regeneration and
repair in a variety of mammals, including humans.

The foregoing and other objects, features and
20 advantages of the present invention will be made more
apparent from the following detailed description of the
invention.

003756-09297
465260" 99/2/99

Brief Description of the Drawings:

The foregoing and other objects and features of this invention, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

Fig. 1(A and B) are photomicrographs illustrating the ability of morphogen (OP-1) to induce transformed neuroblastoma x glioma cells (1A) to redifferentiate to a morphology characteristic of untransformed neurons (1B);

15 Fig. 2A is a dose response curve for the induction of the 180 kDa and 140 kDa N-CAM isoforms in morphogen-treated NG108-15 cells;

Fig. 2B is a photomicrograph of a Western blot of whole cell extracts from morphogen-treated NG108-15 cells with an N-CAM-specific antibody; and

Fig. 3 is a plot of the mean number of cell aggregates counted in 20 randomly selected fields as a function of morphogen concentration.

Fig. 4 is a photomicrograph of an immunoblot demonstrating the presence of OP-1 in human serum.

Detailed Description of the Invention

It now has been discovered that the proteins described herein are effective agents for enhancing the survival of neurons, particularly neurons at risk of dying, and for maintaining neural pathways in a mammal. As described herein, these proteins ("morphogens") are capable of enhancing survival of non-mitotic neurons, stimulating neuronal CAM expression, maintaining the phenotypic expression of differentiated neurons, inducing the redifferentiation of transformed cells of neural origin, and stimulating axonal growth over breaks in neural processes, particularly large gaps in distal axons. The proteins also are capable of providing a neuroprotective effect to alleviate the tissue destructive effects associated with immunologically-related nerve tissue damage. Finally, the proteins may be used as part of a method for monitoring the viability of nerve tissue in a mammal.

Provided below are detailed descriptions of suitable morphogens useful in the methods, compositions and devices of this invention, as well as methods for their administration and application, and numerous, nonlimiting examples which 1) illustrate the suitability of the morphogens and morphogen-stimulating agents described herein as therapeutic agents for maintaining neural pathways in a mammal and enhancing survival of neuronal cells at risk of dying; and 2) provide assays with which to test candidate morphogens and morphogen-stimulating agents for their efficacy.

I. Useful Morphogens

As defined herein a protein is morphogenic if it is capable of inducing the developmental cascade of cellular and molecular events that culminate in the formation of new, organ-specific tissue and comprises at least the conserved C-terminal six cysteine skeleton or its functional equivalent (see supra). Specifically, the morphogens generally are capable of all of the following biological functions in a morphogenically permissive environment: stimulating proliferation of progenitor cells; stimulating the differentiation of progenitor cells; stimulating the proliferation of differentiated cells; and supporting the growth and maintenance of differentiated cells, including the "redifferentiation" of transformed cells. Details of how the morphogens useful in the method of this invention first were identified, as well as a description on how to make, use and test them for morphogenic activity are disclosed in USSN 667,274, filed March 11, 1991 and USSN 752,764, filed August 30, 1991, the disclosures of which are hereby incorporated by reference. As disclosed therein, the morphogens may be purified from naturally-sourced material or recombinantly produced from procaryotic or eucaryotic host cells, using the genetic sequences disclosed therein. Alternatively, novel morphogenic sequences may be identified following the procedures disclosed therein.

Particularly useful proteins include those which comprise the naturally derived sequences disclosed in Table II. Other useful sequences include biosynthetic

5

10

15

20

25

35

of Needleman et al. (1970) J. Mol. Biol., 48:443-453, calculated using the Align Program (DNASTar, Inc.) In the table, three dots indicates that the amino acid in that position is the same as the amino acid in hOP-1.

- 5 Three dashes indicates that no amino acid is present in that position, and are included for purposes of illustrating homologies. For example, amino acid residue 60 of CBMP-2A and CBMP-2B is "missing". Of course, both these amino acid sequences in this region
- 10 comprise Asn-Ser (residues 58, 59), with CBMP-2A then comprising Lys and Ile, whereas CBMP-2B comprises Ser and Ile.

15

TABLE II

20	hOP-1	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val	
	mOP-1	
	hOP-2	...	Arg	Arg	
	mOP-2	...	Arg	Arg	
	DPP	...	Arg	Arg	...	Ser	
	Vgl	Lys	Arg	His	
	Vgr-1	Gly	
25	CBMP-2A	Arg	...	Pro	
	CBMP-2B	...	Arg	Arg	...	Ser	
	BMP3	...	Ala	Arg	Arg	Tyr	...	Lys	...	
	GDF-1	...	Arg	Ala	Arg	Arg	
	60A	...	Gln	Met	Glu	Thr	
30	BMP5	
	BMP6	...	Arg	
		1				5				
35	hOP-1	Ser	Phe	Arg	Asp	Leu	Gly	Trp	Gln	Asp
	mOP-1

00937756-09597
465260-9572680

5	hOP-2	Gln	Leu	...
	mOP-2	Ser	Leu	...
	DPP	Asp	...	Ser	...	Val	Asp	...
	Vgl	Glu	...	Lys	...	Val	Asn
	Vgr-1	Gln	...	Val
	CBMP-2A	Asp	...	Ser	...	Val	Asn	...
	CBMP-2B	Asp	...	Ser	...	Val	Asn	...
	BMP3	Asp	...	Ala	...	Ile	Ser	Glu
	GDF-1	Glu	Val	His	Arg
	60A	Asp	...	Lys	His	...
10	BMP5
	BMP6	Gln
			10					15		
20	hOP-1	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala
	mOP-1
	hOP-2	...	Val	Gln	Ser
	mOP-2	...	Val	Gln	Ser
	DPP	Val	Leu	Asp
	Vgl	...	Val	Gln	Met
	Vgr-1	Lys
	CBMP-2A	Val	Pro	His
	CBMP-2B	Val	Pro	Gln
	BMP3	Ser	...	Lys	Ser	Phe	Asp
25	GDF-1	...	Val	Arg	...	Phe	Leu
	60A	Gly
	BMP5
	BMP6	Lys
30				20					25	
35	hOP-1	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
	mOP-1
	hOP-2	Ser
	mOP-2

265260" 95446630

	CBMP-2A
	CBMP-2B
	BMP3	Ser	Thr	Ile	...	Ser	Ile
	GDF-1	Leu	Val	Leu	Arg	Ala	...
5	60A
	BMP5
	BMP6
		45					50			
10	hOP-1	Val	His	Phe	Ile	Asn	Pro	Glu	Thr	Val
	mOP-1	Asp
	hOP-2	...	His	Leu	Met	Lys	...	Asn	Ala	...
	mOP-2	...	His	Leu	Met	Lys	...	Asp	Val	...
15	DPP	...	Asn	Asn	Asn	Gly	Lys	...
	Vgl	Ser	...	Glu	Asp	Ile
	Vgr-1	Val	Met	Tyr	...
	CBMP-2A	...	Asn	Ser	Val	...	Ser	---	Lys	Ile
	CBMP-2B	...	Asn	Ser	Val	...	Ser	---	Ser	Ile
20	BMP3	...	Arg	Ala**	Gly	Val	Val	Pro	Gly	Ile
	GDF-1	Met	...	Ala	Ala	Ala	...	Gly	Ala	Ala
	60A	Leu	Leu	Glu	...	Lys	Lys	...
	BMP5	Leu	Met	Phe	...	Asp	His	...
	BMP6	Leu	Met	Tyr	...
25			55					60		
	hOP-1	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln
	mOP-1
30	hOP-2	Ala	Lys
	mOP-2	Ala	Lys
	DPP	Ala	Val
	Vgl	...	Leu	Val	Lys
	Vgr-1	Lys
35	CBMP-2A	Ala	Val	Glu

0093756-092597

5	CBMP-2B	Ala	Val	Glu
	BMP3	...	Glu	Val	...	Glu	Lys
	GDF-1	Asp	Leu	Val	...	Ala	Arg
	60A	Arg
	BMP5	Lys
	BMP6	Lys
				65						
										70
10	hOP-1	Leu	Asn	Ala	Ile	Ser	Val	Leu	Tyr	Phe
	mOP-1
	hOP-2	...	Ser	...	Thr	Tyr
	mOP-2	...	Ser	...	Thr	Tyr
	Vgl	Met	Ser	Pro	Met	...	Phe	Tyr
	Vgr-1	Val
15	DPP	...	Asp	Ser	Val	Ala	Met	Leu
	CBMP-2A	...	Ser	Met	Leu
	CBMP-2B	...	Ser	Met	Leu
	BMP3	Met	Ser	Ser	Leu	...	Ile	...	Phe	Tyr
	GDF-1	...	Ser	Pro	Phe	...
	60A	...	Gly	...	Leu	Pro	His
20	BMP5
	BMP6
				75						
										80
25	hOP-1	Asp	Asp	Ser	Ser	Asn	Val	Ile	Leu	Lys
	mOP-1
	hOP-2	...	Ser	...	Asn	Arg
	mOP-2	...	Ser	...	Asn	Arg
	DPP	Asn	...	Gln	...	Thr	...	Val
	Vgl	...	Asn	Asn	Asp	Val	...	Arg
30	Vgr-1	Asn
	CBMP-2A	...	Glu	Asn	Glu	Lys	...	Val
	CBMP-2B	...	Glu	Tyr	Asp	Lys	...	Val
	BMP3	...	Glu	Asn	Lys	Val
	GDF-1	...	Asn	...	Asp	Val	...	Arg
		Val	...	Arg

265260" 9544680

	60A	Leu	Asn	Asp	Glu	Asn
	BMP5
	BMP6	Asn
						85				
5										
	hOP-1	Lys	Tyr	Arg	Asn	Met	Val	Val	Arg	
	mOP-1	
	hOP-2	...	His	Lys	
10	mOP-2	...	His	Lys	
	DPP	Asn	...	Gln	Glu	...	Thr	...	Val	
	Vgl	His	...	Glu	Ala	...	Asp	
	Vgr-1	
	CBMP-2A	Asn	...	Gln	Asp	Glu	
15	CBMP-2B	Asn	...	Gln	Glu	Glu	
	BMP3	Val	...	Pro	Thr	...	Glu	
	GDF-1	Gln	...	Glu	Asp	Asp	
	60A	Ile	...	Lys	
	BMP5	
20	BMP6	Trp	
		90					95			
	hOP-1	Ala	Cys	Gly	Cys	His				
25	mOP-1				
	hOP-2				
	mOP-2				
	DPP	Gly	Arg				
	Vgl	Glu	Arg				
30	Vgr-1				
	CBMP-2A	Gly	Arg				
	CBMP-2B	Gly	Arg				

08937756-093597
465360-9544680

	BMP3	Ser	...	Ala	...	Arg
	GDF-1	Glu	Arg
	60A	Ser
	BMP5	Ser
5	BMP6

100

**Between residues 56 and 57 of BMP3 is a Val residue;
between residues 43 and 44 of GDF-1 lies
the amino acid sequence Gly-Gly-Pro-Pro.

10

As is apparent from the foregoing amino acid
sequence comparisons, significant amino acid changes
can be made within the generic sequences while
15 retaining the morphogenic activity. For example, while
the GDF-1 protein sequence depicted in Table II shares
only about 50% amino acid identity with the hOP1
sequence described therein, the GDF-1 sequence shares
greater than 70% amino acid sequence homology (or
20 "similarity") with the hOP1 sequence, where "homology"
or "similarity" includes allowed conservative amino
acid changes within the sequence as defined by Dayoff,
et al., Atlas of Protein Sequence and Structure vol.5,
supp.3, pp.345-362, (M.O. Dayoff, ed., Nat'l BioMed.
25 Res. Fd'n, Washington D.C. 1979.)

The currently most preferred protein sequences
useful as morphogens in this invention include those
having greater than 60% identity, preferably greater
30 than 65% identity, with the amino acid sequence
defining the conserved six cysteine skeleton of hOP1
(e.g., residues 43-139 of Seq. ID No. 5). These most
preferred sequences include both allelic and species
variants of the OP-1 and OP-2 proteins, including the
35 *Drosophila* 60A protein. Accordingly, in still another

0893746 09397
265660 95/25680

preferred aspect, the invention includes morphogens comprising species of polypeptide chains having the generic amino acid sequence referred to herein as "OPX", which defines the seven cysteine skeleton and
5 accommodates the identities between the various identified mouse and human OP1 and OP2 proteins. OPX is presented in Seq. ID No. 29. As described therein, each Xaa at a given position independently is selected from the residues occurring at the corresponding
10 position in the C-terminal sequence of mouse or human OP1 or OP2 (see Seq. ID Nos. 5-8 and/or Seq. ID Nos. 16-23).

15 II. Formulations and Methods for Administering
Therapeutic Agents

The morphogens may be provided to an individual by any suitable means, preferably directly (e.g., locally, as by injection to a nerve tissue locus) or
20 systemically (e.g., parenterally or orally). Where the morphogen is to be provided parenterally, such as by intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal,
25 intraperitoneal, buccal, rectal, vaginal, intranasal or by aerosol administration, the morphogen preferably comprises part of an aqueous solution. The solution is physiologically acceptable so that in addition to delivery of the desired morphogen to the patient, the
30 solution does not otherwise adversely affect the patient's electrolyte and volume balance. The aqueous medium for the morphogen thus may comprise normal physiologic saline (9.85% NaCl, 0.15M), pH 7-7.4. The aqueous solution containing the morphogen can be made,
35 for example, by dissolving the protein in 50% ethanol

00037756-09997
465250-9574580

containing acetonitrile in 0.1% trifluoroacetic acid (TFA) or 0.1% HCl, or equivalent solvents. One volume of the resultant solution then is added, for example, to ten volumes of phosphate buffered saline (PBS), which further may include 0.1-0.2% human serum albumin (HSA). The resultant solution preferably is vortexed extensively. If desired, a given morphogen may be made more soluble by association with a suitable molecule. For example, association of the mature dimer with the pro domain of the morphogen increases solubility of the protein significantly. In fact, the endogenous protein is thought to be transported in this form. Another molecule capable of enhancing solubility and particularly useful for oral administrations, is casein. For example, addition of 0.2% casein increases solubility of the mature active form of OP-1 by 80%. Other components found in milk and/or various serum proteins also may be useful.

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Sciences (Gennaro, A., ed.), Mack Pub., 1990. Formulations may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity. Biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, polybutyrate, tricalcium phosphate, lactide and lactide/glycolide copolymers, may be useful excipients to control the release of the morphogen in vivo. Other potentially useful parenteral delivery systems for these morphogens

include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or cutric acid for vaginal administration.

Alternatively, the morphogens described herein may be administered orally. Oral administration of proteins as therapeutics generally is not practiced as most proteins are readily degraded by digestive enzymes and acids in the mammalian digestive system before they can be absorbed into the bloodstream. However, the morphogens described herein typically are acid stable and protease-resistant (see, for example, U.S. Pat.No. 4,968,590.) In addition, at least one morphogen, OP-1, has been identified in mammary gland extract, colostrum and 57-day milk. Moreover, the OP-1 purified from mammary gland extract is morphogenically active. Specifically, this protein induces endochondral bone formation in mammals when implanted subcutaneously in association with a suitable matrix material, using a standard in vivo bone assay, such as is disclosed in U.S. Pat.No. 4,968,590. Moreover, the morphogen also is detected in the bloodstream (see Example 9, below). Finally, soluble form morphogen, e.g., mature morphogen associated with the pro domain, is capable of maintaining neural pathways in a mammal (See Examples 4

and 6 below). These findings indicate that oral and parenteral administration are viable means for administering morphogens to an individual. In addition, while the mature forms of certain morphogens described herein typically are sparingly soluble, the morphogen form found in milk (and mammary gland extract and colostrum) is readily soluble, probably by association of the mature, morphogenically active form with part or all of the pro domain of the intact sequence and/or by association with one or more milk components. Accordingly, the compounds provided herein also may be associated with molecules capable of enhancing their solubility in vitro or in vivo.

The compounds provided herein also may be associated with molecules capable of targeting the morphogen or morphogen-stimulating agent to nerve tissue. For example, an antibody, antibody fragment, or other binding protein that interacts specifically with a surface molecule on nerve tissue cells, including neuronal or glial cells, may be used. Useful targeting molecules may be designed, for example, using the single chain binding site technology disclosed, for example, in U.S. Pat. No. 5,091,513.

As described above, the morphogens provided herein share significant sequence homology in the C-terminal active domains. By contrast, the sequences typically diverge significantly in the sequences which define the pro domain. Accordingly, the pro domain is thought to be morphogen-specific. As described above, it is also known that the various morphogens identified to date are differentially expressed in the different tissues. Accordingly, without being limited to any given theory, it is likely that, under natural conditions in the

body, selected morphogens typically act on a given tissue. Accordingly, part or all of the pro domains which have been identified associated with the active form of the morphogen in solution, may serve as
5 targeting molecules for the morphogens described herein. For example, the pro domains may interact specifically with one or more molecules at the target tissue to direct the morphogen associated with the pro domain to that tissue. Accordingly, another useful
10 targeting molecule for targeting morphogen to nerve tissue is part or all of a morphogen pro domain, particularly part or all of the pro domains of OP-1 or GDF-1, both of which proteins are found naturally associated with nerve tissue.

15 Finally, the morphogens or morphogen-stimulating agents provided herein may be administered alone or in combination with other molecules known to be beneficial in maintaining neural pathways, including nerve growth
20 factors and anti-inflammatory agents.

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and
25 carriers. As noted above, such compositions may be prepared for parenteral administration, particularly in the form of liquid solutions or suspensions; for oral administration, particularly in the form of tablets or capsules; or intranasally, particularly in the form of
30 powders, nasal drops, or aerosols.

The compositions can be formulated for parenteral or oral administration to humans or other mammals in therapeutically effective amounts, e.g., amounts which
35 provide appropriate concentrations for a time

08937756-092597
465260-9572680

sufficient to eliminate or reduce the patient's pathological condition, to provide therapy for the neurological diseases and disorders described above, and amounts effective to enhance neural cell survival an/or to protect neurons and neural pathways in anticipation of injury to nerve tissue.

As will be appreciated by those skilled in the art, the concentration of the compounds described in a therapeutic composition will vary depending upon a number of factors, including the dosage of the drug to be administered, the chemical characteristics (e.g., hydrophobicity) of the compounds employed, and the route of administration. The preferred dosage of drug to be administered also is likely to depend on such variables as the type and extent of progression of the neurological disease, the overall health status of the particular patient, the relative biological efficacy of the compound selected, the formulation of the compound excipients, and its route of administration. In general terms, the compounds of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. Typical dose ranges are from about 10 ng/kg to about 1 g/kg of body weight per day; a preferred dose range is from about 0.1 μ g/kg to 100 mg/kg of body weight per day. Optimally, the morphogen dosage given in all cases is between 2-20 μ g of protein per kilogram weight of the patient per day. No obvious OP-1 induced pathological lesions are induced when mature morphogen (e.g., OP-1, 20 μ g) is

00937756-093597

administered daily to normal growing rats for
21 consecutive days. Moreover, 10 μ g systemic
injections of morphogen (e.g., OP-1) injected daily for
10 days into normal newborn mice does not produce any
5 gross abnormalities.

Since the ability of proteins and protein fragments
to penetrate the blood-brain barrier may be related to
their size, lipophilicity or their net ionic charge,
10 suitable modifications of the morphogens may be
formulated (e.g., by substituting
pentafluorophenylalanine for phenylalanine, or by
conjugation to a cationized protein such as albumin) to
increase their transportability across the barrier,
15 using standard methodologies known in the art. See,
for example, Kastin et al., Pharmac. Biochem. Behav.
(1979) 11:713-716; Rapoport et al., Science (1980)
207:84-86; Pardridge et al., (1987) Biochem. Biophys.
Res. Commun. 146:307-313; Riekkinen et al., (1987)
20 Peptides 8:261-265. The efficacy of these functional
analogues may be assessed for example, by evaluating the
ability of these compounds to induce redifferentiation
of transformed cells, or enhance survival of neurons at
risk of dying, as described in the Examples provided
25 herein.

In administering morphogens systemically in the
methods of the present invention, preferably a large
volume loading dose is used at the start of the
30 treatment. The treatment then is continued with a
maintenance dose. Further administration then can be
determined by monitoring at intervals the levels of the
morphogen in the blood.

Where injury to neurons of a neural pathway is induced deliberately as part of, for example, a surgical procedure, the morphogen preferably is provided just prior to, or concomitant with induction of the trauma. Preferably, the morphogen is administered prophylactically in a surgical setting. Optimally, the morphogen dosage given in all cases is between 2-20 μ g of protein per kilogram weight of the patient.

10

Alternatively, an effective amount of an agent capable of stimulating endogenous morphogen levels may be administered by any of the routes described above. For example, an agent capable of stimulating morphogen production and/or secretion from nerve tissue cells may be provided to a mammal, e.g., by direct administration of the morphogen to glial cells associated with the nerve tissue to be treated. A method for identifying and testing agents capable of modulating the levels of endogenous morphogens in a given tissue is described generally herein in Example 13, and in detail in copending USSN 752,859, filed August 30, 1991, the disclosure of which is incorporated herein by reference. Briefly, candidate compounds can be identified and tested by incubating the compound in vitro with a test tissue or cells thereof, for a time sufficient to allow the compound to affect the production, i.e., the expression and/or secretion, of a morphogen produced by the cells of that tissue. Here, suitable tissue or cultured cells of a tissue preferably would comprise neurons and/or glial cells. For example, suitable tissue for testing may include cultured cells isolated from the substantia nigra, adendema glial cells, and the like.

35

0892736-092597

08937756 "062507"
A currently preferred detection means for evaluating the level of the morphogen in culture upon exposure to the candidate compound comprises an immunoassay utilizing an antibody or other suitable binding protein capable of reacting specifically with a morphogen and being detected as part of a complex with the morphogen. Immunoassays may be performed using standard techniques known in the art and antibodies raised against a morphogen and specific for that morphogen. As described herein, morphogens may be isolated from natural-sourced material or they may be recombinantly produced. Agents capable of stimulating endogenous morphogens then may be formulated into pharmaceutical preparations and administered as described herein.

Where the morphogen is to be provided to a site to stimulate axon regeneration, the morphogen preferably is provided to the site in association with a biocompatible, preferably bioresorbable carrier suitable for maintaining a protein at a site in vivo, and through which a neural process may regenerate. A currently preferred carrier also comprises sufficient structure to assist direction of axonal growth. Currently preferred carriers include structural molecules such as collagen, hyaluronic acid or laminin, and/or synthetic polymers or copolymers of, for example, polylactic acid, polyglycolic acid or polybutyric acid. Currently most preferred are carriers comprising tissue extracellular matrix. These may be obtained commercially. In addition, a brain tissue-derived extracellular matrix may be prepared as described in USSN 752,264, incorporated hereinabove by reference, and/or by other means known in the art.

0893756 "0893756"
The currently preferred means for repairing breaks in neural pathways, particularly pathways of the peripheral nervous system, include providing the morphogen to the site as part of a device that includes
5 a biocompatible membrane or casing of a dimension sufficient to span the break and having openings adapted to receive severed nerve ends. The morphogen is disposed within the casing, preferably dispersed throughout a suitable carrier, and is accessible to the
10 severed nerve ends. Alternatively, the morphogen may be adsorbed onto the interior surface of the casing, or otherwise associated therewith. In addition, currently preferred casings have an impermeable exterior surface. The casing acts as a nerve guidance channel, aiding in
15 directing axonal growth. In addition, the casing also protects the damaged nerve from immunologically-related agents which may assist in scar tissue formation. Suitable channel or casing materials include silicone or bioresorbable materials such as collagen, hyaluronic
20 acid, laminin, polylactic acid, polyglycolic acid, polybutyric acid and the like. Additionally, although the nerve guidance channels described herein generally are tubular in shape, it should be evident to those skilled in the art that various alternative shapes may
25 be employed. The lumen of the guidance channels may, for example, be oval or even square in cross section. Moreover the guidance channels may be constructed of two or more parts which may be clamped together to secure the nerve stumps. Nerve endings may be secured
30 to the nerve guidance channels by means of sutures, biocompatible adhesives such as fibrin glue, or other means known in the medical art.

III. Examples

Example 1. Identification of Morphogen-Expressing Tissue

5

Determining the tissue distribution of morphogens may be used to identify different morphogens expressed in a given tissue, as well as to identify new, related morphogens. Tissue distribution also may be used to
10 identify useful morphogen-producing tissue for use in screening and identifying candidate morphogen-stimulating agents. The morphogens (or their mRNA transcripts) readily are identified in different tissues using standard methodologies and minor
15 modifications thereof in tissues where expression may be low. For example, protein distribution may be determined using standard Western blot analysis or immunofluorescent techniques, and antibodies specific to the morphogen or morphogens of interest. Similarly,
20 the distribution of morphogen transcripts may be determined using standard Northern hybridization protocols and transcript-specific probes.

Any probe capable of hybridizing specifically to a
25 transcript, and distinguishing the transcript of interest from other, related transcripts may be used. Because the morphogens described herein share such high sequence homology in their active, C-terminal domains, the tissue distribution of a specific morphogen
30 transcript may best be determined using a probe specific for the pro region of the immature protein and/or the N-terminal region of the mature protein. Another useful sequence is the 3' non-coding region flanking and immediately following the stop codon.
35 These portions of the sequence vary substantially among

08937756-092597

the morphogens of this invention, and accordingly, are specific for each protein. For example, a particularly useful Vgr-1-specific probe sequence is the PvuII-SacI fragment, a 265 bp fragment encoding both a portion of the untranslated pro region and the N-terminus of the mature sequence (see Lyons et al. (1989) PNAS 86:4554-4558 for a description of the cDNA sequence). Similarly, particularly useful mOP-1-specific probe sequences are the BstXI-BglI fragment, a 0.68 Kb sequence that covers approximately two-thirds of the mOP-1 pro region; a StuI-StuI fragment, a 0.2 Kb sequence immediately upstream of the 7-cysteine domain; and the Earl-PstI fragment, an 0.3 Kb fragment containing a portion of the 3'untranslated sequence (See Seq. ID No. 18, where the pro region is defined essentially by residues 30-291.) Similar approaches may be used, for example, with hOP-1 (Seq. ID No. 16) or human or mouse OP-2 (Seq. ID Nos. 20 and 22.)

Using these morphogen-specific probes, which may be synthetically engineered or obtained from cloned sequences, morphogen transcripts can be identified in mammalian tissue, using standard methodologies well known to those having ordinary skill in the art. Briefly, total RNA is prepared from various adult murine tissues (e.g., liver, kidney, testis, heart, brain, thymus and stomach) by a standard methodology such as by the method of Chomczyaski et al. ((1987) Anal. Biochem 162:156-159) and described below. Poly (A)+ RNA is prepared by using oligo (dT)-cellulose chromatography (e.g., Type 7, from Pharmacia LKB Biotechnology, Inc.). Poly (A)+ RNA (generally 15 µg) from each tissue is fractionated on a 1% agarose/formaldehyde gel and transferred onto a Nytran membrane (Schleicher & Schuell). Following the

5
10

15
20
25
30

embryonic tissue. Specifically, northern blots of murine embryos and 6-day post-natal animals shows abundant OP2 expression in 8-day embryos. Expression is reduced significantly in 17-day embryos and is not
5 detected in post-natal animals.

Example 2. Morphogen Localization in the Nervous System

10 Morphogens have been identified in developing and adult rat brain and spinal cord tissue, as determined both by northern blot hybridization of morphogen-specific probes to mRNA extracts from developing and adult nerve tissue (see Example 1, above) and by
15 immunolocalization studies. For example, northern blot analysis of developing rat tissue has identified significant OP-1 mRNA transcript expression in the CNS (USSN 752,764, and Ozkaynak et al. (1991) Biochem. Biophys. Res. Comm., 179:11623 and Ozkaynak, et al.
20 (1992) JBC, in press). GDF-1 mRNA appears to be expressed primarily in developing and adult nerve tissue, specifically in the brain, including the cerebellum and brain stem, spinal cord and peripheral nerves (Lee, S. (1991) PNAS 88: 4250-4254). BMP2B
25 (also referred in the art as BMP4) and Vgr-1 transcripts also have been reported to be expressed in nerve tissue (Jones et al. (1991) Development 111:531-542), although the nerve tissue does not appear to be the primary expression tissue for these genes
30 (Ozkaynak, et al., (1992) JBC in press). Specifically, CBMP2 transcripts are reported in the region of the diencephalon associated with pituitary development, and Vgr-1 transcripts are reported in the anteroposterior axis of the CNS, including in the roof plate of the
35 developing neural tube, as well as in the cells

immediately adjacent the floor plate of the developing neural tube. In older rats, Vgr-1 transcripts are reported in developing hippocampus tissue. In addition, the genes encoding OP-1 and BMP2 originally
5 were identified by probing human hippocampus cDNA libraries.

Immunolocalization studies, performed using standard methodologies known in the art and disclosed
10 in USSN 752,764, filed August 30, 1991, the disclosure of which is incorporated herein, localized OP-1 expression to particular areas of developing and adult rat brain and spinal cord tissue. Specifically, OP-1 protein expression was assessed in adult (2-3 months
15 old) and five or six-day old mouse embryonic nerve tissue, using standard morphogen-specific antisera, specifically, rabbit anti-OP1 antisera, made using standard antibody protocols known in the art and preferably purified on an OP-1 affinity column. The
20 antibody itself was labelled using standard fluorescent labelling techniques, or a labelled anti-rabbit IgG molecule was used to visualize bound OP-1 antibody.

As can be seen in FIG 1A and 1B, immunofluorescence
25 staining demonstrates the presence of OP-1 in adult rat central nervous system (CNS.) Similar and extensive staining is seen in both the brain (1A) and spinal cord (1B). OP-1 appears to be localized predominantly to the extracellular matrix of the grey matter (neuronal
30 cell bodies), distinctly present in all areas except the cell bodies themselves. In white matter, formed mainly of myelinated nerve fibers, staining appears to be confined to astrocytes (glial cells). A similar staining pattern also was seen in newborn rat (10 day
35 old) brain sections.

0893756-092597
265260-9522680

In addition, OP-1 has been specifically localized in the substantia nigra, which is composed primarily of striatal basal ganglia, a system of accessory motor neurons that function is association with the cerebral cortex and corticospinal system. Dysfunctions in this subpopulation or system of neurons are associated with a number of neuropathies, including Huntington's chorea and Parkinson's disease.

OP1 also has been localized at adendema glial cells, known to secrete factors into the cerebrospinal fluid, and which occur around the intraventricular valve, coroid fissure, and central canal of the brain in both developing and adult rat.

Finally, morphogen inhibition in developing embryos inhibits nerve tissue development. Specifically, 9-day mouse embryo cells, cultured in vitro under standard culturing conditions, were incubated in the presence and absence of an OP-1-specific monoclonal antibody prepared using recombinantly produced, purified mature OP-1 and the immunogen. The antibody was prepared using standard antibody production means well known in the art and as described generally in Example 13. After two days, the effect of the antibody on the developing embryo was evaluated by histology. As determined by histological examination, the OP-1-specific antibody specifically inhibits eye lobe formation in the developing embryo. In particular, the diencephalon outgrowth does not develop. In addition, the heart is malformed and enlarged. Moreover, in separate immunolocalization studies on embryo sections with labelled OP-1 specific antibody, the OP-1-specific antibody localizes to neural epithelia.

00937756-092597

The endogenous morphogens which act on neuronal cells may be expressed and secreted by nerve tissue cells, e.g., by neurons and/or glial cells associated with the neurons, and/or they may be transported to the neurons by the cerebrospinal fluid and/or bloodstream. Recently, OP-1 has been identified in the human blood (See Example 9, below). In addition, transplanted Schwann cells recently have been shown to stimulate nerve fiber formation in rat spinal cord, including inducing vascularization and myelin sheath formation around at least some of the new neuronal processes (Bunge (1991) Exp. Neurology 114:254-257.) The regenerative property of these cells may be mediated by the secretion of a morphogen by the Schwann cells.

Example 3. Morphogen Enhancement of Neuronal Cell Survival

The morphogens described herein enhance cell survival, particularly of neuronal cells at risk of dying. For example, fully differentiated neurons are non-mitotic and die in vitro when cultured under standard mammalian cell culture conditions, using a chemically defined or low serum medium known in the art, (see, for example, Charness (1986) J. Biol. Chem. 26:3164-3169 and Freese et al. (1990) Brain Res. 521:254-264.) However, if a primary culture of non-mitotic neuronal cells is treated with a morphogen, the survival of these cells is enhanced significantly. For example, a primary culture of striatal basal ganglia isolated from the substantia nigra of adult rat brain was prepared using standard procedures, e.g., by dissociation by trituration with pasteur pipette of substantia nigra tissue, using standard tissue culturing protocols, and grown in a low serum medium, e.g.,

containing 50% DMEM (Dulbecco's modified Eagle's medium), 50% F-12 medium, heat inactivated horse serum supplemented with penicillin/streptomycin and 4 g/l glucose. Under standard culture conditions, these
5 cells are undergoing significant cell death by three weeks when cultured in a serum-free medium. Cell death is evidenced morphologically by the inability of cells to remain adherent and by changes in their ultrastructural characteristics, e.g., by chromatin
10 clumping and organelle disintegration.

In this example, the cultured basal ganglia were treated with chemically defined medium conditioned with 0.1-100 ng/ml OP-1. Fresh, morphogen-conditioned
15 medium was provided to the cells every 3-4 days. Cell survival was enhanced significantly and was dose dependent upon the level of OP-1 added: cell death decreased significantly as the concentration of OP-1 was increased in cell cultures. Specifically, cells
20 remained adherent and continued to maintain the morphology of viable differentiated neurons. In the absence of morphogen treatment, the majority of the cultured cells dissociated and underwent cell necrosis.

25 Dysfunctions in the basal ganglia of the substantia nigra are associated with Huntington's chorea and parkinsonism in vivo. The ability of the morphogens defined herein to enhance neuron survival indicates that these morphogens will be useful as part of a
30 therapy to enhance survival of neuronal cells at risk of dying in vivo due, for example, to a neuropathy or chemical or mechanical trauma. It is particularly anticipated that these morphogens will provide a useful therapeutic agent to treat neuropathies which affect
35 the striatal basal ganglia, including Huntington's

0003756-092597
265260-954680

chorea and Parkinson's disease. For clinical applications, the morphogen may be administered or, alternatively, a morphogen-stimulating agent may be administered.

5

Example 4. Morphogen-Induced Redifferentiation of Transformed Cells

10 The morphogens described herein also induce
redifferentiation of transformed cells to a morphology
characteristic of untransformed cells. In particular,
the morphogens are capable of inducing
redifferentiation of transformed cells of neuronal
15 origin to a morphology characteristic of untransformed
neurons. The example provided below details morphogen
induced redifferentiation of a transformed human cell
line of neuronal origin, NG105-115. Morphogen-induced
redifferentiation of transformed cells also has been
20 shown in mouse neuroblastoma cells (N1E-115) and in
human embryo carcinoma cells (see copending USSN
752,764, incorporated herein by reference.)

NG108-15 is a transformed hybrid cell line produced
25 by fusing neuroblastoma x glioma cells (obtained from
America Type Tissue Culture, Rockville, MD), and
exhibiting a morphology characteristic of transformed
embryonic neurons, e.g., having a fibroblastic
morphology. Specifically, the cells have polygonal
30 cell bodies, short, spike-like processes and make few
contacts with neighboring cells (see FIG. 1A).
Incubation of NG108-15 cells, cultured in a chemically
defined, serum-free medium, with 0.1 to 300 ng/ml of
OP-1 for four hours induces an orderly, dose-dependent
35 change in cell morphology.

0093756-002597

0893756 092597

In the experiment NG108-15 cells were subcultured on poly-L-lysine coated 6-well plates. Each well contained 40-50,000 cells in 2.5 ml of chemically defined medium. On the third day 2.5 μ l of OP-1 in 60% ethanol containing 0.025% trifluoroacetic was added to each well. OP-1 concentrations of 0-300 ng/ml were tested. Typically, the media was changed daily with new aliquots of OP-1, although morphogenesis can be induced by a single four hour incubation with OP-1. In addition, OP-1 concentrations of 10 ng/ml were sufficient to induce redifferentiation. After one day, hOP-1-treated cells undergo a significant change in their cellular ultrastructure, including rounding of the soma, increase in phase brightness and extension of the short neurite processes. After two days, cells treated with OP-1 begin to form epithelioid sheets, which provide the basis for the growth of multilayered aggregates at three day's post-treatment. By four days, the great majority of OP-1-treated cells are associated in tightly-packed, multilayered aggregates (Fig. 1B). Fig. 2 plots the mean number of multilayered aggregates or cell clumps identified in twenty randomly selected fields from six independent experiments, as a function of morphogen concentration. Forty ng/ml of OP-1 is sufficient for half maximum induction of cell aggregation.

The morphogen-induced redifferentiation occurred without any associated changes in DNA synthesis, cell division, or cell viability, making it unlikely that the morphologic changes were secondary to cell differentiation or a toxic effect of hOP-1. Moreover, the OP-1-induced morphogenesis does not inhibit cell division, as determined by ^3H -thymidine uptake, unlike other molecules which have been shown to stimulate

differentiation of transformed cells, such as butyrate, DMSO, retanoic acid or Forskolin. The data indicate that OP-1 can maintain cell stability and viability after inducing redifferentiation. In addition, the effects are morphogen specific, and redifferentiation is not induced when NG108-15 cells are incubated with 0.1-40 ng/ml TGF- β .

The experiments also have been performed with highly purified soluble morphogen (e.g., mature OP1 associated with its pro domain) which also specifically induced redifferentiation of NG108-15 cells.

The morphogens described herein accordingly provide useful therapeutic agents for the treatment of neoplasias and neoplastic lesions of the nervous system, particularly in the treatment of neuroblastomas, including retinoblastomas, and gliomas. The morphogens themselves may be administered or, alternatively, a morphogen-stimulating agent may be administered.

Example 5. Nerve Tissue Protection from Chemical Trauma

The ability of the morphogens described herein to enhance survival of neuronal cells and to induce cell aggregation and cell-cell adhesion in redifferentiated cells, indicates that the morphogens will be useful as therapeutic agents to maintain neural pathways by protecting the cells defining the pathway from the damage caused by chemical trauma. In particular, the morphogens can protect neurons, including developing neurons, from the effects of toxins known to inhibit

5 The toxic effects of ethanol on developing neurons induces the neurological damage manifested in fetal alcohol syndrome. The morphogens also may protect neurons from the cytotoxic effects associated with excitatory amino acids such as glutamate.

For example, ethanol inhibits the cell-cell adhesion effects induced in morphogen-treated NG108-15 cells when provided to these cells at a concentration of 25-50 mM. Half maximal inhibition can be achieved with 5-10 mM ethanol, the concentration of blood alcohol in an adult following ingestion of a single alcoholic beverage. Ethanol likely interferes with the homophilic binding of CAMs between cells, rather than their induction, as morphogen-induced N-CAM levels are unaffected by ethanol. Moreover, the inhibitory effect is inversely proportional to morphogen concentration. Accordingly, it is envisioned that administration of a morphogen or morphogen-stimulating agent to neurons, particularly developing neurons, at risk of damage from exposure to toxins such as ethanol, may protect these cells from nerve tissue damage by overcoming the toxin's inhibitory effects. The morphogens described herein also are useful in therapies to treat damaged neural pathways resulting from a neuropathy induced by exposure to these toxins.

Example 6. Morphogen-Induced CAM Expression

0893756-092597
The morphogens described herein induce CAM
expression, particularly N-CAM expression, as part of
5 their induction of morphogenesis. CAMs are
morphoregulatory molecules identified in all tissues as
an essential step in tissue development. N-CAMs, which
comprise at least 3 isoforms (N-CAM-180, N-CAM-140 and
N-CAM-120, where "180", "140" and "120" indicate the
10 apparent molecular weights of the isoforms as measured
by polyacrylamide gel electrophoresis) are expressed at
least transiently in developing tissues, and
permanently in nerve tissue. Both the N-CAM-180 and N-
CAM-140 isoforms are expressed in both developing and
15 adult tissue. The N-CAM-120 isoform is found only in
adult tissue. Another neural CAM is L1.

N-CAMs are implicated in appropriate neural
development, including appropriate nuerulation,
20 neuronal migration, fasciculation, and synaptogenesis.
Inhibition of N-CAM production, as by complexing the
molecule with an N-CAM-specific antibody, inhibits
retina organization, including retinal axon migration,
and axon regeneration in the peripheral nervous system,
25 as well as axon synapsis with target muscle cells. In
addition, significant evidence indicates that physical
or chemical trauma to neurons, oncogenic transformation
and some genetic neurological disorders are accompanied
by changes in CAM expression, which alter the adhesive
30 or migratory behavior of these cells. Specifically,
increased N-CAM levels are reported in Huntington's
disease striatum (e.g., striatal basal ganglia), and
decreased adhesion is noted in Alzheimer's disease.

The morphogens described herein can stimulate CAM production, particularly L1 and N-CAM production, including all three isoforms of the N-CAM molecule. For example, N-CAM expression is stimulated significantly in morphogen-treated NG108-15 cells. Untreated NG108-15 cells exhibit a fibroblastic, or minimally differentiated morphology and express only the 180 and 140 isoforms of N-CAM normally associated with a developing cell. Following morphogen treatment these cells exhibit a morphology characteristic of adult neurons and express enhanced levels of all three N-CAM isoforms. Using a similar protocol as described in the example below, morphogen treatment of NG108-15 cells also induced L1 expression.

In this example NG108-15 cells were cultured for 4 days in the presence of increasing concentrations of OP-1 and standard Western blots performed on whole cells extracts. N-CAM isoforms were detected with an antibody which crossreacts with all three isoforms, mAb H28.123, obtained from Sigma Chemical Co., St. Louis, the different isoforms being distinguishable by their different mobilities on an electrophoresis gel. Control NG108-15 cells (untreated) express both the 140 kDa and the 180 kDa isoforms, but not the 120 kDa, as determined by western blot analyses using up to 100 μ g of protein. Treatment of NG108-15 cells with OP-1 resulted in a dose-dependent increase in the expression of the 180 kDa and 140 kDa isoforms, as well as the induction of the 120 kDa isoform. See Fig. 2A and 2B. Fig. 2B is a Western blot of OP1-treated NG108-15 cell extracts, probed with mAb H28.123, showing the induction of all three isoforms. Fig. 2A is a dose response curve of N-CAM-180 and N-CAM-140 induction as a function of morphogen concentration. N-

CAM-120 is not shown in the graph as it could not be quantitated in control cells. However, as is clearly evident from the Western blot in Fig. 2A, N-CAM-120 is induced in response to morphogen treatment. The differential induction of N-CAM 180 and 140 isoforms seen may be because constitutive expression of the 140 isoform is close to maximum.

The increase in N-CAM expression corresponded in a dose-dependent manner with the morphogen induction of multicellular aggregates. Compare Fig. 2A and Fig 3. Fig. 3 graphs the mean number of multilayered aggregates (clumps) counted per 20 randomly selected fields in 6 independent experiments, versus the concentration of morphogen. The induction of the 120 isoform also indicates that morphogen-induced redifferentiation of transformed cells stimulates not only redifferentiation of these cells from a transformed phenotype, but also differentiation to a phenotype corresponding to a developed cell. Standard immunolocalization studies performed with the mAb H28.123 on morphogen-treated cells show N-CAM cluster formation associated with the periphery and processes of treated cells and no reactivity with untreated cells. Moreover, morphogen treatment does not appear to inhibit cell division as determined by cell counting or ³H-thymidine uptake. Finally, known chemical differentiating agents, such as Forskolin and dimethylsulfoxide do not induce N-CAM production.

30

In addition, the cell aggregation effects of OP-1 on NG108-15 cells can be inhibited with anti-N-CAM antibodies or antisense N-CAM oligonucleotides. Antisense oligonucleotides can be made synthetically on a nucleotide synthesizer, using standard means known in

35

0893736-09259

the art. Preferably, phosphorothioate oligonucleotides ("S-oligos") are prepared, to enhance transport of the nucleotides across cell membranes. Concentrations of both N-CAM antibodies and N-CAM antisense
5 oligonucleotides sufficient to inhibit N-CAM induction also inhibited formation of multilayered cell aggregates. Specifically, incubation of morphogen-treated NG108-115 cells with 0.3-3 μ M N-CAM antisense S-oligos, 5-500 μ M unmodified N-CAM antisense oligos,
10 or 10 μ g/ml mAb H28.123 significantly inhibits cell aggregation. It is likely that morphogen treatment also stimulates other CAMs, as inhibition is not complete.

15 The experiments also have been performed with soluble morphogen (e.g., mature OP-1 associated with its pro domain) which also specifically induced CAM expression.

20 The morphogens described herein are useful as therapeutic agents to treat neurological disorders associated with altered CAM levels, particularly N-CAM levels, such as Huntington's chorea and Alzheimers' disease, and the like. In clinical applications, the
25 morphogens themselves may be administered or, alternatively, a morphogen-stimulating agent may be administered.

The efficacy of the morphogens described herein to
30 affect N-CAM expression may be assessed in vitro using a suitable cell line and the methods described herein. In addition to a transformed cell line, N-CAM expression can be assayed in a primary cell culture of neural or glial cells, following the procedures
35 described herein. The efficacy of morphogen treatment

08937756-09597
08937756-09597

on N-CAM expression in vivo may be evaluated by tissue biopsy as described in Example 9, below, and detecting N-CAM molecules with an N-CAM-specific antibody, such as mAb H28.123, or using the animal model described in Example 11.

Alternatively, the level of N-CAM proteins or protein fragments present in cerebrospinal fluid or serum also may be detected to evaluate the effect of morphogen treatment. N-CAM molecules are known to slough off cell surfaces and have been detected in both serum and cerebrospinal fluid. In addition, altered levels of the soluble form of N-CAM are associated with normal pressure hydrocephalus and type II schizophrenia. N-CAM fluid levels may be detected following the procedure described in Example 9 and using an N-CAM specific antibody, such as mAb H28.123.

Example 7. Morphogen-Induced Nerve Gap Repair (PNS)

The morphogens described herein also stimulate peripheral nervous system axonal growth over extended distances allowing repair and regeneration of damaged neural pathways. While neurons of the peripheral nervous system can sprout new processes following injury, without guidance these sproutings typically fail to connect appropriately and die. Where the break is extensive, e.g., greater than 5 or 10 mm, regeneration is poor or nonexistent.

In this example morphogen stimulation of nerve regeneration was assessed using the rat sciatic nerve model. The rat sciatic nerve can regenerate spontaneously across a 5 mm gap, and occasionally

across a 10 mm gap, provided that the severed ends are inserted in a saline-filled nerve guidance channel. In this experiment, nerve regeneration across a 12mm gap was tested.

5

Adult female Sprague-Dawley rats (Charles River Laboratories, Inc.) weighing 230-250 g were anesthetized with intraperitoneal injections of sodium pentobarbital 35 mg/kg body weight). A skin incision was made parallel and just posterior to the femur. The avascular intermuscular plane between vastus lateralis and hamstring muscles were entered and followed to the loose fibroareolar tissue surrounding the sciatic nerve. The loose tissue was divided longitudinally thereby freeing the sciatic nerve over its full extent without devascularizing any portion. Under a surgical microscope the sciatic nerves were transected with microscissors at mid-thigh and grafted with an OP-1 gel graft that separated the nerve stumps by 12 mm. The graft region was encased in a silicone tube 20 mm in length with a 1.5 mm inner diameter, the interior of which was filled a morphogen solution. Specifically, The central 12 mm of the tube consisted of an OP-1 gel prepared by mixing 1 to 5 μ g of substantially pure CHO-produced recombinant OP-1 with approximately 100 μ l of MATRIGELTM (from Collaborative Research, Inc., Bedford, MA), an extracellular matrix extract derived from mouse sarcoma tissue, and containing solubilized tissue basement membrane, including laminin, type IV collagen, heparin sulfate, proteoglycan and entactin, in phosphate-buffered saline. The OP-1-filled tube was implanted directly into the defect site, allowing 4 mm

003736 092597
465260 3522680

on each end to insert the nerve stumps. Each stump was abutted against the OP-1 gel and was secured in the silicone tube by three stitches of commercially available surgical 10-0 nylon through the epineurium,
5 the fascicle protective sheath.

In addition to OP-1 gel grafts, empty silicone tubes, silicone tubes filled with gel only and "reverse" autografts, wherein 12 mm transected segments
10 of the animal's sciatic nerve were rotated 180° prior to suturing, were grafted as controls. All experiments were performed in quadruplicate. All wounds were closed by wound clips that were removed after 10 days. All rats were grafted on both legs. At 3 weeks the
15 animals were sacrificed, and the grafted segments removed and frozen on dry ice immediately. Frozen sections then were cut throughout the graft site, and examined for axonal regeneration by immunofluorescent staining using anti-neurofilament antibodies labeled
20 with flurocein (obtained from Sigma Chemical Co., St. Louis).

Regeneration of the sciatic nerve occurred across the entire 12 mm distance in all graft sites wherein
25 the gap was filled with the OP-1 gel. By contrast, empty silicone tubes and reverse autografts did not show nerve regeneration, and only one graft site containing the gel alone showed axon regeneration.

Example 8. Morphogen-Induced Nerve Gap Repair (CNS)

Following axonal damage in vivo the CNS neurons are
unable to resprout processes. Accordingly, trauma to
5 CNS nerve tissue, including the spinal cord, optic
nerve and retina, severely damages or destroys the
neural pathways defined by these cells. Peripheral
nerve grafts have been used in an effort to bypass CNS
axonal damage. Successful autologous graft repair to
10 date apparently requires that the graft site occur near
the CNS neuronal cell body, and a primary result of CNS
axotomy is neuronal cell death. The efficacy of
morphogens described herein on CNS nerve repair, may be
evaluated using a rat crushed optic nerve model such as
15 the one described by Bignami et al., (1979) Exp. Eye
Res. 28: 63-69, the disclosure of which is incorporated
herein by reference. Briefly, and as described
therein, laboratory rats (e.g., from Charles River
Laboratories, Wilmington, MA) are anesthetized using
20 standard surgical procedures, and the optic nerve
crushed by pulling the eye gently out of the orbit,
inserting a watchmaker forceps behind the eyeball and
squeezing the optic nerve with the forceps for
15 seconds, followed by a 30 second interval and second
25 15 second squeeze. Rats are sacrificed at different
time intervals, e.g., at 48 hours, and at 3, 4, 11, 15
and 18 days after operation. The effect of morphogen
on optic nerve repair can be assessed by performing the
experiment in duplicate and providing morphogen or PBS
30 (e.g., 25 μ l solution, and 25 μ g morphogen) to the
optic nerve, e.g., just prior to the operation,
concomitant with the operation, or at specific times
after the operation.

In the absence of therapy, the surgery induces glial scarring of the crushed nerve, as determined by immunofluorescence staining for glial fibrillary acidic protein (GFA), a marker protein for glial scarring, and by histology. Indirect immunofluorescence on air-dried cryostat sections as described in Bignami et al. (1974) J. Comp. Neur. 153: 27-38, using commercially available antibodies to GFA (e.g., Sigma Chemical Co., St. Louis). Reduced levels of GFA are anticipated in animals treated with the morphogen, evidencing the ability of morphogens to inhibit glial scar formation and to stimulate optic nerve regeneration.

Example 9. Nerve Tissue Diagnostics

Morphogen localization in nerve tissue can be used as part of a method for diagnosing a neurological disorder or neuropathy. The method may be particularly advantageous for diagnosing neuropathies of brain tissue. Specifically, a biopsy of brain tissue is performed on a patient at risk, using standard procedures known in the medical art. Morphogen expression associated with the biopsied tissue then is assessed using standard methodologies, as by immunolocalization, using standard immunofluorescence techniques in concert with morphogen-specific antisera or monoclonal antibodies. Specifically, the biopsied tissue is thin sectioned using standard methodologies known in the art, and fluorescently labelled (or otherwise detectable) antibodies incubated with the tissue under conditions sufficient to allow specific antigen-antibody complex formation. The presence and quantity of complex formed then is detected and compared with a predetermined standard or reference

5
10

15
20
25

30

35

diluted in an appropriate buffer, such as phosphate-buffered saline, and 50 μ l aliquots allowed to absorb to flat bottomed wells in microtitre plates pre-coated with morphogen-specific antibody, and allowed to
5 incubate for 18 hours at 4°C. Plates then may be washed with a standard buffer and incubated with 50 μ l aliquots of a second morphogen-specific antibody conjugated with a detecting agent, e.g., biotin, in an appropriate buffer, for 90 minutes at room temperature.
10 Morphogen-antibody complexes then may be detected using standard procedures.

Alternatively, a morphogen-specific affinity column may be created using, for example, morphogen-specific
15 antibodies adsorbed to a column matrix, and passing the fluid sample through the matrix to selectively extract the morphogen of interest. The morphogen then is eluted. A suitable elution buffer may be determined empirically by determining appropriate binding and
20 elution conditions first with a control (e.g., purified, recombinantly-produced morphogen.) Fractions then are tested for the presence of the morphogen by standard immunoblot, and confirmed by N-terminal sequencing. Morphogen concentrations in serum or other
25 fluid samples then may be determined using standard protein quantification techniques, including by spectrophotometric absorbance or by quantitation by ELISA or RIA antibody assays. Using this procedure, OP-1 has been identified in serum.

30

OP-1 was detected in human serum using the following assay. A monoclonal antibody raised against mammalian, recombinantly produced OP-1 using standard immunology techniques well described in the art and
35 described generally in Example 13, was immobilized by

0093756-09297

passing the antibody over an activated agarose gel
(e.g., Affi-GelTM, from Bio-Rad Laboratories, Richmond,
CA, prepared following manufacturer's instructions),
and used to purify OP-1 from serum. Human serum then
5 was passed over the column and eluted with 3M
K-thiocyanate. K-thiocyanate fractions then were
dialyzed in 6M urea, 20mM PO₄, pH 7.0, applied to a C8
HPLC column, and eluted with a 20 minute, 25-50%
acetonitrile/0.1% TFA gradient. Mature, recombinantly
10 produced OP-1 homodimers elute between 20-22 minutes.
Fractions then were collected and tested for the
presence of OP-1 by standard immunoblot. Fig. 4 is an
immunoblot showing OP-1 in human sera under reducing
and oxidized conditions. In the figure, lanes 1 and 4
15 are OP-1 standards, run under oxidized (lane 1) and
reduced (lane 4) conditions. Lane 5 shows molecular
weight markers at 17, 27 and 39 kDa. Lanes 2 and 3 are
human sera OP-1, run under oxidized (lane 2) and
reduced (lane 3) conditions.

20
Morphogens may be used in diagnostic applications
by comparing the quantity of morphogen present in a
body fluid sample with a predetermined reference value,
with fluctuations in fluid morphogen levels indicating
25 a change in the status of nerve tissue. Alternatively,
fluctuations in the level of endogenous morphogen
antibodies may be detected by this method, most likely
in serum, using an antibody or other binding protein
capable of interacting specifically with the endogenous
30 morphogen antibody. Detected fluctuations in the
levels of the endogenous antibody may be used as
indicators of a change in tissue status.

0093756 009397
265260 9522680

Example 10. Alleviation of Immune Response-Mediated
Nerve Tissue Damage

265260-95746680

5 The morphogens described herein may be used to
alleviate immunologically-related damage to nerve
tissue. Details of this damage and the use of
morphogens to alleviate this injury are disclosed in
copening USSN 753,059, filed August 30, 1991, the
disclosure of which is incorporated herein. A primary
10 source of such damage to nerve tissue follows hypoxia
or ischemia-reperfusion of a blood supply to a neural
pathway, such as may result from an embolic stroke, or
may be induced during a surgical procedure. As
described in USSN 753,059, morphogens have been shown
15 to alleviate damage to myocardial tissue following
ischemia-reperfusion of the blood supply to the tissue.
The effect of morphogens on alleviating
immunologically-related damage to nerve tissue may be
assessed using methodologies and models known to those
20 skilled in the art and described below.

For example, the rabbit embolic stroke model
provides a useful method for assessing the effect of
morphogens on tissue injury following cerebral
25 ischemia-reperfusion. The protocol disclosed below is
essentially that of Phillips et al. (1989) Annals of
Neurology 25:281-285, the disclosure of which is herein
incorporated by reference. Briefly, white New England
rabbits (2-3kg) are anesthetized and placed on a
30 respirator. The intracranial circulation then is
selectively catheterized by the Seldinger technique.
Baseline cerebral angiography then is performed,
employing a digital substration unit. The distal
internal carotid artery or its branches then is
35 selectively embolized with 0.035 ml of 18-hour-aged

autologous thrombus. Arterial occlusion is documented by repeat angiography immediately after embolization. After a time sufficient to induce cerebral infarcts (15 minutes or 90 minutes), reperfusion is induced by administering a bolus of a reperfusion agent such as the TPA analogue FB-FB-CF (e.g., 0.8 mg/kg over 2 minutes).

The effect of morphogen on cerebral infarcts can be assessed by administering varying concentrations of morphogens, e.g., OP-1, at different times following embolization and/or reperfusion. The rabbits are sacrificed 3-14 days post embolization and their brains prepared for neuropathological examination by fixing by immersion in 10% neutral buffered formalin for at least 2 weeks. The brains then are sectioned in a coronal plane at 2-3 mm intervals, numbered and submitted for standard histological processing in paraffin, and the degree of nerve tissue necrosis determined visually. Morphogen-treated animals are anticipated to reduce or significantly inhibit nerve tissue necrosis following cerebral ischemia-reperfusion in the test animals as determined by histology comparison with nontreated animals.

Example 11. Animal Model for Assessing Morphogen Efficacy In Vivo

The in vivo activities of the morphogens described herein also are assessed readily in an animal model as described herein. A suitable animal, preferably exhibiting nerve tissue damage, for example, genetically or environmentally induced, is injected intracerebrally with an effective amount of a morphogen in a suitable therapeutic formulation, such as

phosphate-buffered saline, pH 7. The morphogen preferably is injected within the area of the affected neurons. The affected tissue is excised at a subsequent time point and the tissue evaluated morphologically and/or by evaluation of an appropriate biochemical marker (e.g., by morphogen or N-CAM localization; or by measuring the dose-dependent effect on a biochemical marker for CNS neurotrophic activity or for CNS tissue damage, using for example, glial fibrillary acidic protein as the marker. The dosage and incubation time will vary with the animal to be tested. Suitable dosage ranges for different species may be determined by comparison with established animal models. Presented below is an exemplary protocol for a rat brain stab model.

Briefly, male Long Evans rats, obtained from standard commercial sources, are anesthetized and the head area prepared for surgery. The calvariae is exposed using standard surgical procedures and a hole drilled toward the center of each lobe using a 0.035K wire, just piercing the calvariae. 25 μ l solutions containing either morphogen (e.g., OP-1, 25 μ g) or PBS then is provided to each of the holes by Hamilton syringe. Solutions are delivered to a depth approximately 3 mm below the surface, into the underlying cortex, corpus callosum and hippocampus. The skin then is sutured and the animal allowed to recover.

Three days post surgery, rats are sacrificed by decapitation and their brains processed for sectioning. Scar tissue formation is evaluated by immunofluorescence staining for glial fibrillary acidic protein, a marker protein for glial scarring, to qualitatively determine

the degree of scar formation. Glial fibrillary acidic protein antibodies are available commercially, e.g., from Sigma Chemical Co., St. Louis, MO. Sections also are probed with anti-OP-1 antibodies to determine the presence of OP-1. Reduced levels of glial fibrillary acidic protein are anticipated in the tissue sections of animals treated with the morphogen, evidencing the ability of morphogens to inhibit glial scar formation and stimulate nerve regeneration.

10

Example 12. In Vitro Model for Evaluating Morphogen Species Transport Across the Blood-Brain Barrier.

15 Described below is an in vitro method for evaluating the facility with which selected morphogen species likely will pass across the blood-brain barrier. A detailed description of the model and protocol are provided by Audus et al. (1987) Ann. N.Y. Acad. Sci. 507:9-18, the disclosure of which is
20 incorporated herein by reference.

Briefly, microvessel endothelial cells are isolated from the cerebral gray matter of fresh bovine brains. Brains are obtained from a local slaughter house and transported to the laboratory in ice cold minimum essential medium (MEM) with antibiotics. Under sterile conditions the large surface blood vessels and meninges are removed using standard dissection procedures. The
25 cortical gray matter is removed by aspiration, then minced into cubes of about 1mm. The minced gray matter then is incubated with 0.5% dispase (BMB, Indianapolis, IN) for 3 hours at 37° C in a shaking water bath. Following the 3 hour digestion, the mixture is
30 concentrated by centrifugation (1000 x g for 10 min.),

0893756-09597
265260-9574680

then resuspended in 13% dextran and centrifuged for 10 min. at 5800 x g. Supernatant fat, cell debris and myelin are discarded and the crude microvessel pellet resuspended in 1 mg/ml collagenase/dispase and
5 incubated in a shaking water bath for 5 hours at 37° C. After the 5-hour digestion, the microvessel suspension is applied to a pre-established 50% Percoll gradient and centrifuged for 10 min at 1000 x g. The band
10 containing purified endothelial cells (second band from the top of the gradient) is removed and washed two times with culture medium (e.g., 50% MEM/50% F-12 nutrient mix). The cells are frozen (-80° C.) in medium containing 20% DMSO and 10% horse serum for later use.

15

After isolation, approximately 5×10^5 cells/cm² are plated on culture dishes or 5-12 mμ pore size polycarbonate filters that are coated with rat collagen and fibronectin. 10-12 days after seeding the cells,
20 cell monolayers are inspected for confluency by microscopy.

Characterization of the morphological, histochemical and biochemical properties of these cells
25 has shown that these cells possess many of the salient features of the blood-brain barrier. These features include: tight intercellular junctions, lack of membrane fenestrations, low levels of pinocytotic activity, and the presence of gamma-glutamyl
30 transpeptidase, alkaline phosphatase, and Factor VIII antigen activities.

The cultured cells can be used in a wide variety of experiments where a model for polarized binding or
35 transport is required. By plating the cells in

009377W6-09997

multi-well plates, receptor and non-receptor binding of both large and small molecules can be conducted. In order to conduct transendothelial cell flux measurements, the cells are grown on porous polycarbonate membrane filters (e.g., from Nucleopore, Pleasanton, CA). Large pore size filters (5-12 μ) are used to avoid the possibility of the filter becoming the rate-limiting barrier to molecular flux. The use of these large-pore filters does not permit cell growth under the filter and allows visual inspection of the cell monolayer.

Once the cells reach confluency, they are placed in a side-by-side diffusion cell apparatus (e.g., from Crown Glass, Sommerville, NJ). For flux measurements, the donor chamber of the diffusion cell is pulsed with a test substance, then at various times following the pulse, an aliquot is removed from the receiver chamber for analysis. Radioactive or fluorescently-labelled substances permit reliable quantitation of molecular flux. Monolayer integrity is simultaneously measured by the addition of a non-transportable test substance such as sucrose or inulin and replicates of at least 4 determinations are measured in order to ensure statistical significance.

Example 13. Screening Assay for Candidate Compounds which Alter Endogenous Morphogen Levels

Candidate compound(s) which may be administered to affect the level of a given morphogen may be found using the following screening assay, in which the level of morphogen production by a cell type which produces measurable levels of the morphogen is determined with and without incubating the cell in culture with the

compound, in order to assess the effects of the compound on the cell. This can be accomplished by detection of the morphogen either at the protein or RNA level. A more detailed description also may be found
5 in USSN 752,861, incorporated hereinabove by reference.

13.1 Growth of Cells in Culture

Cell cultures of kidney, adrenals, urinary bladder,
10 brain, or other organs, may be prepared as described widely in the literature. For example, kidneys may be explanted from neonatal or new born or young or adult rodents (mouse or rat) and used in organ culture as whole or sliced (1-4 mm) tissues. Primary tissue
15 cultures and established cell lines, also derived from kidney, adrenals, urinary, bladder, brain, mammary, or other tissues may be established in multiwell plates (6 well or 24 well) according to conventional cell culture techniques, and are cultured in the absence or presence
20 of serum for a period of time (1-7 days). Cells may be cultured, for example, in Dulbecco's Modified Eagle medium (Gibco, Long Island, NY) containing serum (e.g., fetal calf serum at 1%-10%, Gibco) or in serum-deprived medium, as desired, or in defined medium (e.g.,
25 containing insulin, transferrin, glucose, albumin, or other growth factors).

Samples for testing the level of morphogen production includes culture supernatants or cell
30 lysates, collected periodically and evaluated for OP-1 production by immunoblot analysis (Sambrook et al., eds., 1989, Molecular Cloning, Cold Spring Harbor Press, Cold Spring Harbor, NY), or a portion of the cell culture itself, collected periodically and used to
35 prepare polyA⁺ RNA for RNA analysis. To monitor de

0893756-09397
265260-9572680

novo OP-1 synthesis, some cultures are labeled according to conventional procedures with an ^{35}S -methionine/ ^{35}S -cysteine mixture for 6-24 hours and then evaluated to OP-1 synthesis by conventional immunoprecipitation methods.

13.2 Determination of Level of Morphogenic Protein

In order to quantitate the production of a morphogenic protein by a cell type, an immunoassay may be performed to detect the morphogen using a polyclonal or monoclonal antibody specific for that protein. For example, OP-1 may be detected using a polyclonal antibody specific for OP-1 in an ELISA, as follows.

1 $\mu\text{g}/100 \mu\text{l}$ of affinity-purified polyclonal rabbit IgG specific for OP-1 is added to each well of a 96-well plate and incubated at 37°C for an hour. The wells are washed four times with 0.167M sodium borate buffer with 0.15 M NaCl (BSB), pH 8.2, containing 0.1% Tween 20. To minimize non-specific binding, the wells are blocked by filling completely with 1% bovine serum albumin (BSA) in BSB and incubating for 1 hour at 37°C . The wells are then washed four times with BSB containing 0.1% Tween 20. A $100 \mu\text{l}$ aliquot of an appropriate dilution of each of the test samples of cell culture supernatant is added to each well in triplicate and incubated at 37°C for 30 min. After incubation, $100 \mu\text{l}$ biotinylated rabbit anti-OP-1 serum (stock solution is about 1 mg/ml and diluted 1:400 in BSB containing 1% BSA before use) is added to each well and incubated at 37°C for 30 min. The wells are then washed four times with BSB containing 0.1% Tween 20. $100 \mu\text{l}$ streptavidin-alkaline (Southern Biotechnology Associates, Inc. Birmingham, Alabama, diluted 1:2000 in

adjuvant and is given subcutaneously. The second injection contains 50 μ g of OP-1 in incomplete adjuvant and is given intraperitoneally. The mouse then receives a total of 230 μ g of OP-1 (amino acids 307-431 in SEQ ID NO:5) in four intraperitoneal injections at various times over an eight month period. One week prior to fusion, both mice are boosted intraperitoneally with 100 μ g of OP-1 (307-431) and 30 μ g of the N-terminal peptide (Ser₂₉₃-Asn₃₀₉-Cys) conjugated through the added cysteine to bovine serum albumin with SMCC crosslinking agent. This boost was repeated five days (IP), four days (IP), three days (IP) and one day (IV) prior to fusion. The mouse spleen cells are then fused to myeloma (e.g., 653) cells at a ratio of 1:1 using PEG 1500 (Boeringer Mannheim), and the cell fusion is plated and screened for OP-1-specific antibodies using OP-1 (307-431) as antigen. The cell fusion and monoclonal screening then are according to standard procedures well described in standard texts widely available in the art.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

adjuvant and is given subcutaneously. The second injection contains 50 μ g of OP-1 in incomplete adjuvant and is given intraperitoneally. The mouse then receives a total of 230 μ g of OP-1 (amino acids 307-431 in SEQ ID NO:5) in four intraperitoneal injections at various times over an eight month period. One week prior to fusion, both mice are boosted intraperitoneally with 100 μ g of OP-1 (307-431) and 30 μ g of the N-terminal peptide (Ser₂₉₃-Asn₃₀₉-Cys) conjugated through the added cysteine to bovine serum albumin with SMCC crosslinking agent. This boost was repeated five days (IP), four days (IP), three days (IP) and one day (IV) prior to fusion. The mouse spleen cells are then fused to myeloma (e.g., 653) cells at a ratio of 1:1 using PEG 1500 (Boehringer Mannheim), and the cell fusion is plated and screened for OP-1-specific antibodies using OP-1 (307-431) as antigen. The cell fusion and monoclonal screening then are according to standard procedures well described in standard texts widely available in the art.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.